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SALT TASTE THRESHOLD OF HUMANS

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Results of self-selection experiments showed that under a wide variety of circumstances rats have the ability to regulate their sodium chloride intake according to their salt needs. Thus, adrenalectomized rats, which as the result of the excessive loss of sodium chloride in the urine usually die within 5 to 15 days, when given free access to salt, will ingest sufficient amounts to keep themselves alive and free from symptoms of insufficiency. Some rats ingested 10 times as much salt after adrenalectomy as before (Richter, 1936; Richter and Eckert, 1938). Pregnant and lactating rats also increased their salt intake very sharply, apparently to take care of the increasing salt needs of the mother and of the developing fetuses and nursing young (Richter and Barelare, 1938). Normal rats, given a wide selection of purified substances, fats, carbohydrates, proteins, vitamins, and essential minerals, including salt, ingested salt in an amount very close to that which McCollum found to be necessary for normal growth and health (Richter, Holt and Barelare, 1938).

Further, it was reported that not only do adrenalectomized rats have an increased appetite for salt, but also a lowered taste threshold for salt (Richter, 1939). Rats receiving the standard McCollum diet were kept separately in cages and given access to 2 graduated inverted bottles. In the beginning both bottles contained distilled water. Records were taken until the daily intake from each bottle became fairly constant. This rarely required more than 6 to 10 days. Then 1 bottle was filled with a salt solution of an estimated subliminal taste concentration. Each day thereafter the concentration of the salt solution was increased in very small steps. It was found that with concentrations of 0.055 per cent, or 1 part of salt to 2,000 parts of water, normal rats began to drink more salt solution and less water. This concentration was taken to be the threshold.

Adrenalectomized rats made this distinction with concentrations of 0.003 per cent, or 1 part of salt to 33,000 parts of water.

The results of these studies indicated that taste thresholds for salt and possibly for other substances might help in the detection of glandular and other deficiencies. With this in mind, studies have been started on taste thresholds of rats and humans for various nutritional substances. The present study deals with the salt taste thresholds of normal human subjects.

In the past many studies have been made on salt taste thresholds of normals largely for academic purposes, having little relation to the rôle differences in threshold might play on dietary selections.

The numerous measurements of the salt threshold of normals that had been made gave widely differing results. A few of these are listed below:

	Concentration of salt solution in per cent
Venables (1887)	0.1
Bailey and Nichols (1888)	0.05
Zuntz (1892)	0.10
Kiesow (1894)	0.25
Heymans (1899)	0.25
Hänig (1901)	0.34
Renqvist (1919)	0.05–0.174
v. Skramlik (1926)	0.05–0.49
Darley and Doan (1936)	0.185

We now have a method which we feel gives more constant and reliable results and which is very similar to that used on rats.

METHODS. We tried out four different methods before an accurate measure of the threshold was obtained. A short account of each method is given below.

In the first or so-called "drop method" we placed 3 drops of the salt solution from a medicine dropper on the middle of the subject's protruded tongue. We started with a concentration of salt solution which was estimated to fall well below the threshold, and with each successive test gave a slightly higher concentration. We used 13 solutions with concentrations ranging from 0.05 to 0.4 per cent.

All of the previous workers except Renqvist failed to state whether by threshold they meant the concentration at which the subjects distinguished salt solution from distilled water, or the concentration at which they first recognized a salt taste. For this reason, our subjects received instructions to state 1, when they first recognized a difference between the salt solution and water, and 2, when they first recognized any definite taste.

Seventeen normal adult subjects, medical students and laboratory assistants, were tested with this method.

The method had several serious disadvantages. First, it was difficult always to place the 3 drops on the same relative area of the tongue in all

the subjects; and secondly, due to the small amount of the salt solution as compared with the saliva, the solution became diluted so quickly that judgment had to be made almost instantaneously.

In a second series of observations using the "swallow method" we gave 10 cc. of salt solution in a dram glass in each trial. Thus, the solution came into contact with all parts of the mouth and tongue in each individual. The subjects received instructions to taste and swallow the solution. In these experiments we used 11 solutions with concentrations ranging from 0.04 to 0.30 per cent.

Twenty-four adults served as subjects with this method.

It was found that this method also had a serious shortcoming. Since the subjects received only salt solutions in increasing concentrations and no distilled water, their judgment was limited to comparison of the taste of one salt solution with that of the foregoing solution of a slightly lower concentration. It thus became very difficult to state when a change in taste had occurred.

This difficulty was eliminated by a third or so-called "choice method." The subjects received 2 dram glasses, each filled at the outset with approximately 10 cc. of distilled water. The subjects received instructions to drink the fluid in each glass and to state whether or not they had the same taste. Then with each successive trial, one glass was filled with salt solution beginning, as heretofore, with subliminal concentrations, while the other glass was filled with distilled water. Thus, the subjects could compare each concentration of salt solution with distilled water. We used 15 solutions with concentrations ranging from 0.01 to 0.225 per cent.

With this method ("choice method" no. 1) we measured thresholds of 28 adult subjects.

This method turned out to have a minor shortcoming. The difficulty arose from the fact that by emptying one glass and then the other, the subject did not have sufficient opportunity to compare the tastes of the two fluids.

In a fourth method ("choice method" no. 2) the subject received instructions to sample the fluids of each glass as often as he wanted, until he felt satisfied in regard to the taste of each. This is essentially the same method as is employed by the rats in choosing between two solutions. The 13 concentrations we used are listed below:

Concentrations of sodium chloride in per cent

0.005	0.05
0.007	0.06
0.009	0.07
0.01	0.08
0.02	0.09
0.03	0.10
0.04	

In order to eliminate complications which might arise from extraneous influences, we blindfolded the subjects and placed the glasses in their hands, interchanging them frequently and in an irregular order.

Fifty-three adult subjects (40 medical students, 4 doctors, 9 laboratory assistants) with an average age of 23 years were tested by this method. The experiments were conducted in a quiet room, free from disturbing stimuli, especially distracting smells.

RESULTS. *Drop method.* The average concentration at which the 17 subjects first recognized a difference between distilled water and salt solution was 0.135 per cent, and the average concentration at which they first

TABLE 1

Thresholds

	TASTE DIFFERENCE	RANGE OF VARIATION	SALT TASTE	RANGE OF VARIATION
Drop method (17)	0.135	0.045–0.225	0.192	0.120–0.350
Swallow method (24)	0.047	0.015–0.150	0.167	0.040–0.400
Choice method no. 1 (28)	0.037	0.007–0.080	0.080	0.030–0.300
Choice method no. 2 (53):				
Mean	0.016	0.007–0.060	0.087	0.020–0.250
Median	0.010		0.065	

TABLE 2

Expressions used to describe sub-threshold taste sensations and frequency of use

	SUBJECTS		SUBJECTS		SUBJECTS		SUBJECTS
Sweet	20	Bicarbonate	2	Soapy	1	Orange juice	1
Bitter	7	Basic	1	Straw	1	Woody	1
Sour	6	Acid	1	Magnesium	1	Potass. chloride	1
Alkaline	2	Slippery	1	Oily	1	Vanilla	1

recognized the taste of salt was 0.192 per cent. These results agreed fairly well with those reported by most of the previous workers.

Swallow method. The second or "swallow method" gave considerably lower thresholds. The average concentration at which the 24 subjects distinguished between salt solution and distilled water was 0.047 per cent, and the average concentration at which they first recognized the salt taste was 0.167 per cent. These values were lower than any of those reported previously.

Choice method no. 1. With this method, by which the subject alternately tasted salt solution and distilled water, the threshold for the salt taste decreased to a still lower level. The average concentration at which 28

subjects first distinguished salt solution from distilled water was 0.037 per cent, and the average concentration at which they first recognized salt was 0.080 per cent.

Choice method no. 2. With this method, which made possible a thorough comparison between the salt solution and the distilled water, the concentration first recognized by 53 subjects as being different from distilled water averaged 0.016 per cent, with a median of 0.010 per cent; and the concentration at which they first recognized the salt taste averaged 0.087 per cent, with a median of 0.065 per cent.

These readings were definitely lower and also more constant than were obtained with any of the other methods.

Table 1 summarizes the results of all four methods.

REMARKS. It is noteworthy that concentrations of salt solutions below the salt taste threshold elicited a variety of taste sensations described by 16 different terms. Table 2 lists the times and the number of subjects that used each term. "Sweet" and "bitter" were most frequently used.

In order to determine whether smoking habits influence the salt taste threshold, we listed each of the subjects as a non, moderate, or heavy smoker. We found no correlation between smoking habits and the thresholds. We made certain, however, that the subjects had not smoked immediately before the tests were made.

DISCUSSION. According to our results normal humans are able to distinguish salt solution from distilled water in average concentrations of 0.016 per cent, but in some instances as low as 0.009 per cent and 0.007 per cent. They first definitely recognize a salt taste in average concentrations of 0.065 per cent, in some instances as low as 0.03 and 0.02 per cent.

These values are considerably lower than those reported by all of the previous workers.

Comparisons of the taste thresholds of rats and humans brings out several interesting facts. The concentration at which rats first distinguished between distilled water and salt solution, 0.05 per cent, coincided fairly closely with the concentration at which normal humans first recognized a salt taste. This threshold, however, was definitely above that at which humans first recognized a difference between salt solution and water. It would seem likely, therefore, that rats may also recognize a difference in lower concentrations but do not start to drink more salt solution until they can recognize the taste of salt.

Wedell (1936) reported that rats and humans have a very similar taste threshold for quinine. He based his conclusions on his observations that the quinine threshold of 14 rats averaged 0.000718 per cent and on Blakeslee's observations (1932) that the lowest quinine threshold recorded in 21 humans was 0.000300 per cent.

Whether humans with salt deficiencies such as are seen in Addison's disease show a lowered taste threshold we do not know. Darley and Doan reported a lowered salt taste threshold (0.092 per cent as compared to 0.185 per cent calculated for normals) in a white, 20 year old patient who showed remarkable craving for salt ever since childhood and ate enormous quantities of salt. At autopsy this patient's adrenals escaped examination. Extensive pulmonary arteriosclerosis was found.

Finally, we might mention the possibility that the threshold may be determined at least in part by the salt content of the saliva. It is conceivable that a greatly lowered salt content of the saliva in adrenalectomized rats may play an important part in accounting for the lowering of the salt taste threshold.

SUMMARY

1. A description was presented of a method for measuring salt taste thresholds in humans which gives much lower values than have been reported previously and is practically identical with the method used for salt taste threshold studies in rats.

2. The average concentration at which 53 adult humans first recognized a difference in taste between salt solution and distilled water was 0.016 per cent with a median of 0.010 per cent.

3. The average concentration at which they first definitely recognized the taste of salt was 0.087 per cent with a median of 0.065 per cent.

4. The threshold at which humans first recognized the salt taste closely agreed with the threshold found in rats.

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MODIFICATION OF ADRENALIN INTOXICATION BY ADRENALECTOMY

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Intoxication by massive doses of adrenalin, the so-called "adrenalin shock," has been described by many authors—by Pellacani (1) as early as 1879, in 1883 by Foa and Pellacani (2), and most recently by Parkins, Swingle, Taylor and Hays (3). Schultz (4) has given much information on the toxicity of adrenalin, and Erlanger and Gasser (5) and Freeman (6) have studied its circulatory manifestations. The large literature of the subject cannot be cited here in full, but most of the remaining important titles may be found in the above papers.

Among the given causes of death, those most frequently mentioned are ventricular fibrillation, asphyxia, heart failure, and circulatory collapse. Aside from possible direct effects on the respiratory center or the heart, there seem to be two types of lethal concomitants: first, pulmonary congestion with edema or hemorrhage subsequent to acute cardiac dilatation or left ventricular failure (7, 8); and, secondly, a shock-like failure of the circulation—with low blood pressure and decrease in blood volume (6)—ascribed to prolonged vasoconstriction (5) and capillary atony (3). The first is found when death occurs soon after injection, appears to be asphyxial, and is generally correlated with the rapid administration of large amounts of adrenalin in a single dose; while the second accompanies death after one or several hours without violent dyspneic symptoms, and is more likely to follow the slow infusion of adrenalin over a longer period. We shall call these two types *acute* and *subacute*.

The lowered resistance of adrenalectomized animals to all sorts of toxic or other damaging agents is well known. Very little has been reported, however, with regard to adrenalin intoxication. Selye (9) states that "adrenalectomized animals are extremely sensitive to adrenaline" but does not give details. Parkins *et al.* (3) revived adrenalectomized dogs from "adrenalin shock" by the injection of cortical hormone. During experiments upon the production of lens opacities in rats, one of us (C. tum Suden) noticed that a dose of adrenalin which would characteristically produce "acute" death in normal rats was more likely to produce

the "subacute" type in adrenalectomized rats. A further investigation of this point is here reported.

METHODS. Single doses of adrenalin chloride, 1 to 1000 (Parke, Davis) were administered by intraperitoneal injection. The reactions of the following groups of non-fasted, young adults (average weight of males 250 grams, of females 170 grams) of our inbred strain of albino rats were compared: normal; adrenalectomized (one to two weeks after operation); transplants (adrenalectomized rats maintained in health and vigor by either three months old autoplastic or homoplastic cortical grafts, or by rapidly proliferating "accessory" cortical tissue soon after operation);

TABLE 1

DESCRIPTION	NO.	ADRENALIN	ACUTE DEATHS 0-60 MIN.			SUBACUTE DEATHS 60-720 MIN.			DELAYED DEATHS 12-24 hr.			SURVIVALS	
			No.	Mean time	Per cent	No.	Mean time	Per cent	No.	Mean time	Per cent	No.	Per cent
		mgm. per 100 gm.		*min.*			*min.*			*hrs.*			
Normal	59	0.2-0.3	32	27.8	54.2	5	277	8.5	2	18±	3.4	20	33.9
Normal	45	0.4, 0.5	40	15.1	89.0	2	129	4.4	0	0	0	3	6.6
Adrenalectomized	64	0.2-0.3	0	0	0	26	338	40.6	12	18±	18.8	26	40.6
Adrenalectomized	29	0.4, 0.5	11	16.0	37.9	15	322	51.7	3	14±	10.4	0	0
Transplants	35	0.2-0.3	34	22.0	97.1	0	0	0	0	0	0	1	2.9
Transplants	6	0.4	6	16.3	100.0	0	0	0	0	0	0	0	0
Fasted normal	28	0.3	26	21.2	92.9	0	0	0	0	0	0	2	7.1
Fasted adrenalectomized	12	0.3	4	23.0	33.3	4	243	33.3	0	0	0	4	33.3
Normal + histamine 20-50 mgm./100 gm.	34	0.2-0.35	6	61.5	17.6	20	173	58.9	0	0	0	8	23.5
Normal + histamine 50 mgm./100 gm.	26	0.5	8	40.1	30.8	9	144	34.6	4	18±	15.4	5	19.2
Young rats (6 to 8 weeks) less than 100 grams													
Normal	15	0.3, 0.5	12	11.4	80.0	1	78	6.7	0	0	0	2	13.3
Normal + histamine 50 mgm./100 gm.	12	0.3, 0.5	2	35.0	16.6	1	91	8.3	2	24±	16.3	7	58.1
Normal + histamine 100 mgm./100 gm.	9	0.5, 1.0	4	60.0	44.0	2	?	22.0	0	0	0	3	33.3

and normal with histamine (ergamine acid phosphate, Burroughs Wellcome) in single intraperitoneal injections given ten to twenty minutes before the test dose of adrenalin. Although there were indications that the females were consistently more resistant to adrenalin than the males (slightly increased survival time) these differences were not great enough to be proved statistically significant.

RESULTS. *Normal rats.* A gross summary of the results is presented in table 1. From 0.4 to 0.5 mgm. of adrenalin per 100 grams body weight was lethal in less than half an hour for the majority of normal rats (89 per cent), and is probably above what might be considered the minimal lethal dose for this substance. A smaller critical dose, from 0.2 to 0.3

mgm. per 100 grams, which was lethal for about two-thirds of a series, was chosen as a basis of comparison. Most of the rats (82 per cent) that were killed by this dose died acutely within an hour with marked dyspnea and asphyxial convulsions. Autopsy showed right cardiac dilatation, and acute pulmonary congestion with edema and hemorrhage. Microscopic examination of the lungs confirmed the above, showing extensive rhexis of alveolar walls.

Since the reactions to toxic doses of adrenalin vary considerably with numerous factors (see Schultz, 4) it is difficult to compare the results of different workers, but it seems that the rat is about ten times more resistant to adrenalin than most other species studied.

Adrenalectomized rats. Of the adrenalectomized rats receiving from 0.2 to 0.3 mgm. of adrenalin, about the same proportion died within 24 hours (60 per cent) as in the normal series; but none died acutely, the majority surviving for five and a half hours or more. After a short period of mild malaise they became progressively weaker, cold, and comatose, with terminal convulsions (characteristic of hypoglycemia). Dyspnea and asphyxia were absent, and autopsy and microscopic examination showed practically normal lungs. Even with the higher dosages (0.4 to 0.5 mgm.) 62.1 per cent died with subacute symptoms. The shift from the acute to the subacute type of death appears soon after removal of the adrenal cortex (at least 60 hours, 10 cases).

Although the percentages of survivals over twenty-four hours in the normal and adrenalectomized series were about the same, the adrenalectomized—after a temporary recovery—died during the following two weeks at a time after operation less than that characteristic of death from adrenal insufficiency for the same age and strain. This contrasts with the speed and permanency of the recovery of the normals, and undoubtedly reflects the impaired mobilization of reserves (e.g., carbohydrate) in adrenalectomized animals subjected to stress. It also permits an interpretation of previous statements that the adrenalectomized animal is "extremely sensitive to adrenalin." The results show, however, that adrenalectomy increases resistance to the specific acute, asphyxial effects of adrenalin.

Susceptibility to adrenalin is increased by fasting (6 to 18 hrs.) in both normal and adrenalectomized rats. Because of the inadequate carbohydrate mechanism of the latter (see below) the decrease in survival time is to be expected, but the reason for the increased mortality of the normals is not so obvious.

Blood pressure. In two normal and three adrenalectomized rats under urethane anesthesia, carotid blood pressures were recorded by the mercury manometer. After intraperitoneal injection of 0.3 mgm. adrenalin per 100 grams, the blood pressure rose promptly to around 150 mm. Hg. The normal rats maintained this raised level until acute dyspnea began

(27 and 50 min.), when the pressure dropped to zero within two to five minutes and death ensued. The adrenalectomized rats maintained the raised pressure for about half an hour. There was then a slow, progressive fall to zero during the following one to two hours, i.e., a "shock-like" reaction.

Transplants. The shift from the acute to the subacute type of death following adrenalectomy was not due to the loss of the adrenal medulla alone. This was strikingly shown by the fact that rats having cortical tissue, but no medulla, were more susceptible than the normal, 97 per cent of those receiving from 0.2 to 0.3 mgm. of adrenalin dying acutely in less than an hour. Not only the percentage but the mean time of acute deaths was significantly different (18.8 vs. 34.1 min. for the lower dosage, 0.2 mgm.). Autopsy findings were the same as those in the normals. Minute "accessory" masses in recently operated rats were as effective as large, long established grafts.

Normal rats with histamine. Pre-treatment with histamine so prolongs the survival time of normal rats receiving from 0.2 to 0.5 mgm. per 100 grams of adrenalin, that the tabulation of results resembles that of the adrenalectomized series. The optimum amount of histamine seemed to be about 100 times that of the adrenalin. Moreover a significant number survived doses of 0.5 mgm. This was most strikingly demonstrated in the young rat of less than 100 grams body weight, where doses of 0.5 and 1.0 mgm. of adrenalin were antagonized by histamine. Blood sugar findings of less than 40 mgm. per cent at death, as well as the gross and microscopic appearance of the lungs (practically normal), confirmed the impression that a shift from the acute to the subacute type of death was produced by histamine as well as by adrenalectomy.

Blood sugar changes. In several cases (3 to 10) from each group the blood sugar changes were roughly followed by the Folin-Malmros micro-method. In the normal and in the transplant, heart blood taken just before acute death from 0.3 mgm. adrenalin showed a hyperglycemia of 265 and 190 mgm. per cent respectively. Fed adrenalectomized rats showed a sharp rise during the first hour above the pre-adrenalin level (103 mgm. per cent) to an average maximum of only 174 mgm. per cent (11 cases), but at death their blood sugar averaged less than 55 mgm. per cent (9 cases). Normal rats pre-treated with histamine showed a similar terminal blood sugar (3 cases). Since the method does not exclude the non-glucose reducing substances, it may be inferred that convulsive levels were attained as indicated by the gross symptoms. In the less resistant normal and adrenalectomized *fasted* rats, including the adrenalectomized rats which died in less than thirty minutes, the blood sugar levels at death showed the same striking divergence. Thus the carbohydrate changes during adrenalin intoxication in rats having cortical tissue (hyperglycemic) and

those without (hypoglycemic) still further differentiate the mechanisms operating in acute and subacute death. The influence of histamine also permits speculation as to its rôle in inducing the subacute type during adrenal insufficiency.

Discussion. Cortical insufficiency alters the organism so that the normal asphyxial reaction to adrenalin intoxication is not only decreased in frequency of occurrence, and abated in intensity, but is practically eliminated after certain critical doses which elicit an acute fatal response in normal animals. Under these conditions a subacute reaction of the "shock" type is revealed, similar in many respects to a precipitated adrenal insufficiency syndrome. The reasons for this alteration are obscure.

Although the response of the adrenalectomized rats was similar to that seen in normal animals after the slow infusion of adrenalin (5), delayed absorption of the drug is not an adequate explanation for their difference from the normals. Initial blood pressures still within normal range, and promptness in the rise after injection of adrenalin—as well as the fact that experiments were done at a time after operation before marked insufficiency and a presumably sluggish circulation had appeared—do not entail a sufficient degree of delayed absorption to make an intraperitoneal injection comparable to slow infusion.

One might invoke the "adaptation energy" concept of Selye (10), who has shown that various damaging stimuli ("alarms") increase the non-specific resistance of rats at first (during the "alarm reaction") and decrease it later when the specific resistance is still high. He has also shown that adrenalin lung edema is prevented by pre-treatment with any one of a variety of damaging agents (11). In our experiments, however, the resistance to the asphyxial reaction was still high at a time after operation when non-specific resistance should have been low. To apply the conception, adrenalin intoxication would have to be regarded as fundamentally the same as the original damage of adrenal removal, or cortical insufficiency as a continuous "alarm." Furthermore, the conception does not elucidate the exact mechanisms involved.

Among the factors that might be so altered by cortical lack as to operate in the shift from the acute to the subacute syndrome are vasomotor reactions, water balance, capillary permeability, carbohydrate mechanism, and reaction of bronchial musculature. Changes in any of these could conceivably oppose the progress of pulmonary edema. The possibility of the accumulation of histamine-like substances, especially in the lungs, should not be overlooked, since histamine treatment likewise produces the shift.

As for the "transplanted" rats, we have previously shown (12) that cortical transplants do not function as efficiently in sudden emergencies as do the normal glands. Possibly the increased susceptibility to ad-

renalin is the outcome of their generally reduced efficiency plus the lack of medullary activity.

SUMMARY

After single massive, intraperitoneal doses of adrenalin (from 0.2 to 0.5 mgm. per 100 grams body weight) the majority of normal rats succumbing died acutely within one hour. These showed asphyxial symptoms from acute pulmonary congestion with hemorrhage and edema. Blood pressure and blood sugar levels remained markedly elevated until death. The survivors recovered rapidly and permanently.

Following adrenalectomy, similar doses of adrenalin killed about the same proportion, but in not less than three to twenty-four hours. This subacute death was characterized by a slow, progressive fall of blood pressure to zero, a decline of blood sugar to a convulsive level, and an absence of pulmonary involvement. The survival time of those that recovered after 24 hours was less than that expected in adrenalectomized rats of the strain used.

In rats having cortical tissue but no medulla the acute symptoms were more severe than in the normal. Fasting likewise increased the susceptibility of both normal and adrenalectomized rats.

Pre-treatment with histamine produced a shift from the acute to the subacute type of death—a shift similar to that caused by adrenalectomy.

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THE EFFECT OF RESECTION OF THE OLFACTORY, GUSTATORY AND TRIGEMINAL NERVES ON WATER DRINKING IN DOGS WITHOUT AND WITH DIABETES INSIPIDUS

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In common human experience the urge to drink water is associated with a localized sensation of dryness in the mouth and throat. Some physiologists (Cannon, 1918; Gregersen, 1938) identify this sensation as the urge to drink water.

Recently it has been shown (Bellows, 1939) that the disappearance of the urge to drink is effected by at least two supplementary factors in dogs. One factor operates in the mouth and pharynx above the level of an esophageal fistula in the neck. The other factor is effective in the gastrointestinal tract below the level of the fistula.

An effort has now been made to determine whether the urge to drink may be abolished or modified by eliminating some of the possible pathways by which localized sensations about the buccal region may be conveyed to the central nervous system. This problem has been approached before. Longet (1868) severed the glossopharyngeal, the lingual and the vagus nerves on both sides in dogs and observed that they drank as usual after eating. Cannon pointed out that all the branches of the trigemini were not cut.

In the present investigation the voluntary intake of water was observed in 3 groups of dogs: *Group 1.* Dogs in which the gustatory sense was believed abolished by division of the glossopharyngeal nerves and chordae tympani bilaterally. *Group 2.* Dogs in which all sensations except taste were believed abolished from the buccal cavity by division of the trigeminal nerves bilaterally. *Group 3.* Dogs whose olfactory sense was believed abolished by resection of the olfactory tracts. In each group the dogs were observed under two conditions: *a*, during a period of normal water intake, and *b*, during the polyposia [< Gr., πολύ, much, + ποσια, drink. Unusually copious and frequent drinking] of diabetes insipidus.

METHODS. Dissections upon cadavers and trial operations in vivo were performed to perfect the surgical technic of the several denervating procedures.

Group 1. Taste. The chorda tympani was resected on each side along with the

lingual nerve intraorally. The mucous membrane over the medial surface of the coronoid process of the mandible was incised above the last molar tooth. This exposed the lingual nerve at the anterior border of the internal pterygoid muscle, which is peripheral to the point where the chorda tympani joins it. A section of the nerve was removed.

The glossopharyngeal nerve was divided extracranially on each side near its emergence from the skull. An incision was made in the neck along the border of the mandible. Dissection was made anterior to the parotid gland, and between the mandible and the posterior belly of the digastric muscle. The glossopharyngeal nerve was identified caudad and medial to the superior cornu of the hyoid bone, and cephalad to the hypoglossal nerve, which was identified by stimulation. The glossopharyngeal nerve was divided as near the skull as possible, and the peripheral end was sutured into the subcutaneous tissues.

Autopsies were not made on the 2 animals of this group because operative identification of the nerves was more satisfactory than post-mortem identification. Dog 1A lived for 6 weeks and dog 2A was sacrificed 34 weeks after total denervation.

Group 2. Trigemini. The posterior root of each trigeminal nerve was divided intracranially. A separate procedure was required for each side, and 6 weeks elapsed between procedures. A vertical incision was made on the side of the head. The zygoma was resected, and the temporal muscle was split and retracted. An area of temporal bone 2.5 cm. in diameter was removed. The dura was opened and the temporal lobe was elevated to expose the petrous bone. The dura over the gasserian ganglion was incised and extended posteriorly to expose the posterior root. The trigeminal canal in the petrous bone was enlarged, and the root was avulsed. Muscle, fascia and skin were closed. Two dogs comprise this group. Dog 2A lived for 8 weeks and dog 2B for 54 weeks after the final denervating procedure.

When the animals were killed the heads were fixed with 10 per cent formalin. The cranial bones were entirely removed from the brains, and the trigeminal nerves were inspected. A section of the pons including the nerve and ganglion was removed, and serial sections were cut through the axis of the nerve. Sections were stained with hematoxylin and eosin, Bodian's (1936) nerve fiber stain, and the myelin sheath stain of Smith and Quigley (1937).

In dog 2A there was complete absence of innervation by the trigeminal nerves. On one side the posterior root was macroscopically separated from the pons. The divided end of the root was covered with connective tissue, and the cavity in the pons left by the avulsed root was also filled with connective tissue. On the opposite side the root appeared intact. Microscopically there was interruption of the axones at the junction of the central or glial segment and the peripheral or neurilemmal segment (Tarlov, 1937). In the central fragment of the root the axones were degenerated.

In dog 2B there was incomplete loss of trigeminal innervation. There was complete separation of the root on one side with the interposition of connective tissue between the fragments. On the opposite side only the lateral half of the root had been divided. According to anatomical studies by Spiller and Frazier (1933) and others, this would produce anesthesia in the entire distribution of the mandibular nerve, and in part of the distribution of the maxillary nerve. This would include most if not all of the buccal mucous membrane on one side. The dog did not react to painful stimuli to either side of the face. There was a faint corneal reflex on the side of incomplete nerve section.

Group 3. Smell. In order to resect the olfactory tracts it was necessary to make a vertical transverse section of the entire brain posterior to the olfactory bulbs. Through a median incision the roof and floor of each frontal sinus was removed.

The dura was opened on each side of the falx cerebri. The frontal lobes and olfactory tracts were resected with the electro-cautery, the falx cerebri being left intact. The dura over the floor of the skull was thoroughly cauterized to insure destruction of the nervus terminalis. Dura and skin were closed. Four dogs comprise this group. Dogs 3A and 3B lived for 9 and 4 weeks respectively, dog 3C for 3 years, and dog 3D for 28 months after resection of the olfactory tracts.

The heads of 2 dogs, 3A and 3D, after fixation with 10 per cent formalin, were sectioned in the median sagittal plane. At the site of the brain resection a thick, dense transverse septum of fibrous tissue had formed, which separated the olfactory bulbs and frontal lobes from the remainder of the brain.

Hypothalamic injury. The technic of transbuccal operations for producing diabetes insipidus has been described (Bellows and Van Wagenen, 1938). In dogs 1B, 3A, and 3D the infundibular stalk was compressed with a silver clip. In dogs 2A,

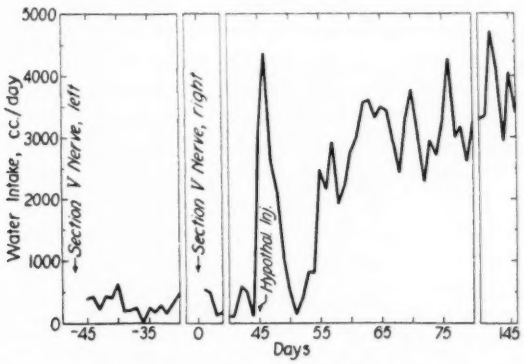

Fig. 1. Daily voluntary water intakes of dog 2B before and after bilateral division of the trigemini. On day 0 denervation was complete. On day 45 the tuber cinereum was cauterized. Typical diabetes insipidus followed: temporary phase, days 45 to 50 inclusive; remission; permanent phase began day 53 and reached steady stage day 63.

2B, 3B, and 3C the tuber cinereum was cauterized. In dog 1A a silver clip was placed on the infundibular stalk by the subtemporal route.

The dogs were kept in metabolism cages. They were fed a diet of kitchen leavings daily. Voluntary water intakes were recorded once a day for continuous periods before and after denervation. In groups 1 and 2, where two or more procedures were required to secure total denervation, the first period of observation followed the first procedure (fig. 1).

Daily water intakes were recorded continuously after hypothalamic injury, but only certain observations are reported. Diabetes insipidus occurs in two phases separated by a remission in which water intake is normal (fig. 1). In the temporary phase polyposia becomes maximal the first or second day after hypothalamic injury, then gradually diminishes and disappears by about the seventh day. The maximal intake and the average intake of the temporary phase are reported. Polyposia of the permanent phase increases from about the 10th to the 21st days after hypothalamic injury and then becomes steady. Average intakes of the permanent phase were computed after polyposia had become steady.

OBSERVATIONS. The graphic protocol of dog 2B, shown in figure 1, is typical of the protocols of all the animals of all groups. All results are summarized in table 1.

Group 1. Section of all nerves believed to mediate taste led to no significant change in water drinking before hypothalamic injury. After injury the customary onset and course of the polyposia of diabetes insipidus took place.

TABLE 1

Average daily water intakes for continuous periods before and after each sensory loss and during diabetes insipidus

DOG	WT.	BEFORE TOTAL SENSORY LOSS		AFTER TOTAL SENSORY LOSS						
		Before hypothalamic injury				During diabetes insipidus				
						Temporary phase			Permanent phase	
		Aver.	Days	Aver.	Days	Max.	Aver.	Days	Aver.	Days
	kgm.	*cc.*		*cc.*		*cc.*	*cc.*		*cc.*	
1A	15.1	258	16	269	8	2,750	1,221	5	434	14
1B	11.6	549	20	—	—	3,800	1,868	7	1,638	91
2A	10.4	—	—	147	19	2,000	1,125	6	Died	
2B	15.5	304	15	339	4	4,385	1,973	7	3,554	85
				288	5					
3A	23.0	475	4	425	8	11,700	3,179	7	1,498	34
3B	—	—	—	490	20	Died				
3C	12.3	233	8	319	8	1,300	642	7	825	17

DOG	WT.	DURING PERMANENT PHASE OF DIABETES INSIPIDUS					
		Before loss of smell				After loss of smell	
		Month	Aver.	Month	Aver.	Month	Aver.
			cc.		*cc.*		*cc.*
3D	12.4	1st	2,312	4th	1,446	7th	983

The relatively small daily water intakes in the permanent phase of dog 1A can be ascribed to the method of producing diabetes insipidus, for the use of the silver clip often results in slight or diminishing polyposia in the permanent phase (Bellows and Van Wagenen, 1938). In dog 1B a silver clip was similarly utilized, but the polyposia corresponded in amount to that usually observed in non-denervated dogs. It is evident that the urge to drink water is not dependent on the nerves of taste alone.

Group 2. Bilateral section of the trigeminal nerves neither changed water drinking before hypothalamic injury, nor modified the customary polyposia of diabetes insipidus after injury. During the period of observa-

tion after denervation dog 2A was unable to eat because of extreme stiffness of the masticatory muscles. It is believed that the enforced fasting was responsible for the small daily water intakes.

Dog 2B, whose trigeminal nerve on one side was incompletely divided, was included because certainly half and probably the entire buccal cavity was believed deprived of innervation, yet no modification in water drinking was observed. Dog 2A, whose believed anesthesia of the buccal cavity was complete, demonstrated that the urge to drink water is not incited solely by any of the sensations mediated by the trigemini.

Group 3. After olfactory tract resection there were temporary irregularities in water drinking. The records for these days were omitted in computing averages. Dog 3A drank 100 cc. of water or less daily for 3 days. Dog 3B stood much of the time for 8 days with his forefeet in the water-can and splashed water about with his feet and muzzle. In this way he drank or splashed greater quantities of water than he formerly drank. Dog 3C drank no water on the day of operation. Dog 3D, whose olfactory tracts were divided in the 6th month of the permanent phase of diabetes insipidus, drank an average of only 535 cc. of water a day for 10 days with a range from 100 cc. to 825 cc. a day.

The temporary diminution of the water intake observed in dogs 3A, 3C and 3D may have been caused by fasting, which has been observed to diminish the water intake of normal dogs (Gregersen, 1938), and of dogs with diabetes insipidus (Curtis, 1924). The animals were observed to refuse food or to eat sparingly for short periods after olfactory loss. The quantities eaten and the duration of these fasting periods were not recorded. The behavior of dog 3B suggests a temporary disturbance or perversion of the urge to drink as a consequence of the believed loss of smell.

When the daily water intakes of each dog again become uniform, they did not differ appreciably in amount from those which prevailed before the believed loss of smell. After hypothalamic injury, polyposia developed in its usual phases and quantities. The polyposia of dog 3D was less after resection of the olfactory tracts than it had been previously. Diabetes insipidus had been produced with the silver clip, and it is suggested that the diminution in the polyposia was not the result of believed olfactory loss, but was the continuation of the decrement apparent before resection.

It is evident that believed loss of the olfactory sense alone has no permanent effect upon water drinking.

Other effects of the denervations deserve mention. It was not observed that believed loss of taste changed eating habits. The dogs in whom the trigemini were divided generally developed keratitis and corneal opacity. Blindness and anesthesia of the muzzle made the dogs awkward in getting about. Normal eating habits were resumed after post-operative stiffness had disappeared from the masticatory muscles.

Believed loss of the olfactory sense changed the dogs' temperments and eating habits. Dog 3B was wide-eyed and fearful. He viciously attacked anything that touched him, whether it was an attendant's hand, a stick, or another dog. When handled he became maniacal. Dog 3A was not vicious but kept his tail between his legs. His only reaction to another dog was to follow him. Dog 3D, long accustomed to cage life, appeared to show simultaneously alarm, surprise and fear. These traits never wholly disappeared. The temperment of dog 3C remained unchanged. All the dogs ignored food at first. As they resumed eating they became untidy. They scattered food about the cage. They chewed food and ejected it. They took wood or paper into the mouth and ejected it only after chewing it. They ate meat moistened with ether, from which a normal dog recoiled. Loss of the frontal lobes of the brain may have contributed to the changes in temperament.

COMMENT. Reichert and Poth (1933) and others have found that the glossopharyngeal nerve is chiefly sensory in man. Its unilateral intracranial section produces unilateral loss of sensation over the uvula, soft palate, posterior pharyngeal wall from the eustachian tube to the epiglottis (including the posterior aspect), over the lateral pharyngeal wall and tonsillar region to the anterior faucial pillar, and over the posterior third of the tongue where taste also is lost. The sensory distribution of the glossopharyngeal nerve doubtless adjoins that of the trigeminal nerve in the nasal and buccal cavities.

A localized sensation of dryness in these areas has been identified with the urge to drink (loc. cit.). One proof of this hypothesis will rest upon the ability to remove the urge to drink by denervation of the areas to which the localized sensation is referred. It has been shown that producing believed anesthesia of either the trigeminal or the glossopharyngeal area in dogs has not abolished or modified water drinking. It seems possible to conclude that 1, the urge to drink water is not removed by denervation of the mouth or pharynx, because 2, localized sensation in these cavities is not the only origin of the urge to drink.

The urge to drink is dispelled by ingesting an amount of water equal to the deficit (Adolph, 1939). Water balance is thus maintained in part by precise quantitative regulation of intake. Dogs in which either the trigeminal or the glossopharyngeal nerves had been divided presumably maintained water balance after denervation as precisely as before. After hypothalamic injury, polyposias were equal to those observed in non-denervated dogs. Quantitatively the regulation of water intake remained intact in spite of believed loss of part of the sensory area identified with intake regulation. Believed anesthesia of both the trigeminal and glossopharyngeal nerve areas was not produced in the same dog.

The vital importance of water suggests that the urge to drink is of such

a nature that it does not disappear while the need for water exists, and that it is not removed by other means than water ingestion. The result of its loss would be fatal, since the ability to regulate water balance would be lost, and vital tissues would have been mortally injured by the factors effecting its loss.

The aid of Dr. E. F. Adolph in preparing this report is gratefully acknowledged.

SUMMARY

Water drinking was observed in 3 groups of dogs; in (1) the sense of taste was believed abolished, and anesthesia of the pharynx was believed obtained; in (2) the sensations mediated by the trigeminal nerves were believed abolished; and in (3) the sense of smell was believed abolished. In each group water drinking was observed in *a*, the normal state, and in *b*, the state of diabetes insipidus.

It was observed that the deprivation of dogs with respect to these senses did not abolish or alter water drinking in normal amounts or in the excessive amounts of diabetes insipidus. The urge to drink may not be identified with any one of the nervous pathways that were interrupted, or with any single one of the types of sensations that they mediate.

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LYMPHATIC ABSORPTION FROM THE NASOPHARYNX[1]

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A spontaneous flow of lymph from cannulated superficial lymphatics seldom occurs in an anesthetized dog. In these quiescent animals there is ordinarily no physiological activity capable of forcing lymph through lymphatic vessels. In order to obtain samples of lymph from a cannulated vessel, the usual procedure, consequently, has been to massage the surface of the body over a lymphatic trunk. This method has definite disadvantages. Lymph is forced intermittently into the cannula, and the amount obtained over a given period of time depends on the rate and force of the massage. In addition to the uneven flow, the composition of lymph may be altered by massage of lymph glands situated in the draining lymphatic pathway. It is thus impossible to maintain standard conditions of lymph flow or of lymph content throughout an experiment.

White, Field and Drinker (1933) overcame these complicating factors by conducting experiments on animals not under a general anesthetic. They cannulated limb lymphatics of dogs under local anesthesia and obtained a spontaneous flow of lymph during periods of graded activity. The method of local anesthesia, however, is not satisfactory in situations involving more extensive experimental procedures.

Lymphatic absorption from the nasopharynx and the rôle of the cervical lymphatic pathway are matters of growing moment in view of our increasing knowledge of the significance of viruses in causing nervous and respiratory disease. Drinker and his associates have found that when a solution of a colloidal dye, such as trypan blue or T-1824 (Yoffey and Drinker, 1938), or a solution of egg or of serum albumin (Yoffey, Sullivan and Drinker, 1938) is dropped into the nose of an anesthetized animal, these substances pass through the nasopharyngeal epithelium to the underlying lymphatics and appear in the draining cervical lymphatic vessels. Schulz, Warren and Drinker (1938) found the same to be true in rabbits receiving

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intranasal doses of type III pneumococci. These experiments indicate a surprising versatility of lymphatic absorption from the nasopharynx.

In order to study this question more fully, a standardized method for producing a flow of cervical lymph in anesthetized animals has been developed. The method consists of regular passive motion of the head, which causes an even flow of lymph without the use of massage and without the disadvantages inherent in the massage technique. Because of the ease and success with which lymph may be secured by this new method, and because it can readily be coupled with perfusion of the nasopharynx, we believe the method to have possibilities of wide application in future studies on factors influencing lymphatic function. The present paper describes the method and reports the results, obtained by its use, of experiments on lymphatic absorption from the nasopharynx.

METHOD. Figure 1 is a diagrammatic representation of the apparatus used to secure passive motion of the head with simultaneous collection of cervical lymph. The letters in italics in the following description refer to corresponding letters of the diagram. Dogs were used in the experiments but the method may be adapted for other animals. The dogs were anesthetized by an intravenous or intraperitoneal injection of nembutal, and the right and left cervical lymphatics then cannulated low in the neck, *E*. A granule of heparin, placed in the cannulae on the loop of a fine wire, prevented clotting. A tracheal cannula, *D*, was inserted for respiration. Ringer's solution (20–25 cc. per kilogram of body weight), given intravenously from a burette, *G*, connected to a cannula in one of the femoral veins, insured an adequate supply of fluid in the body.

A double length of twine, *F*, was tied to the snout, and attached to an electrically driven crank, *H*, as indicated in the diagram. The occipital portion of the head rested in the circular depression of a sloping wooden platform on the animal board. The head was held in position by three rubber bands, *B*, stretched from the snout to stationary metal uprights. Rotation of the crank (ten times per minute), together with the elasticity of the rubber bands, produced a slow regular flexion and extension of the head. This is indicated by dotted lines in figure 1.

In order to determine the amount of lymphatic absorption of fluid from the nasopharynx, Ringer's solution or, in a few cases, distilled water was perfused through this region. The inflow tube consisted of a small, vaseline-covered catheter, *A*, placed in one nostril and attached by rubber tubing to a constant temperature reservoir. The esophagus was ligated and the perfusate allowed to escape through an outflow tube, *C*, tied into the trachea, anterior to the tracheal cannula. Thermometers situated in the inflow and outflow tubes indicated the temperature of the perfusion fluid. A thermometer was placed in the mouth, its tip touching the soft

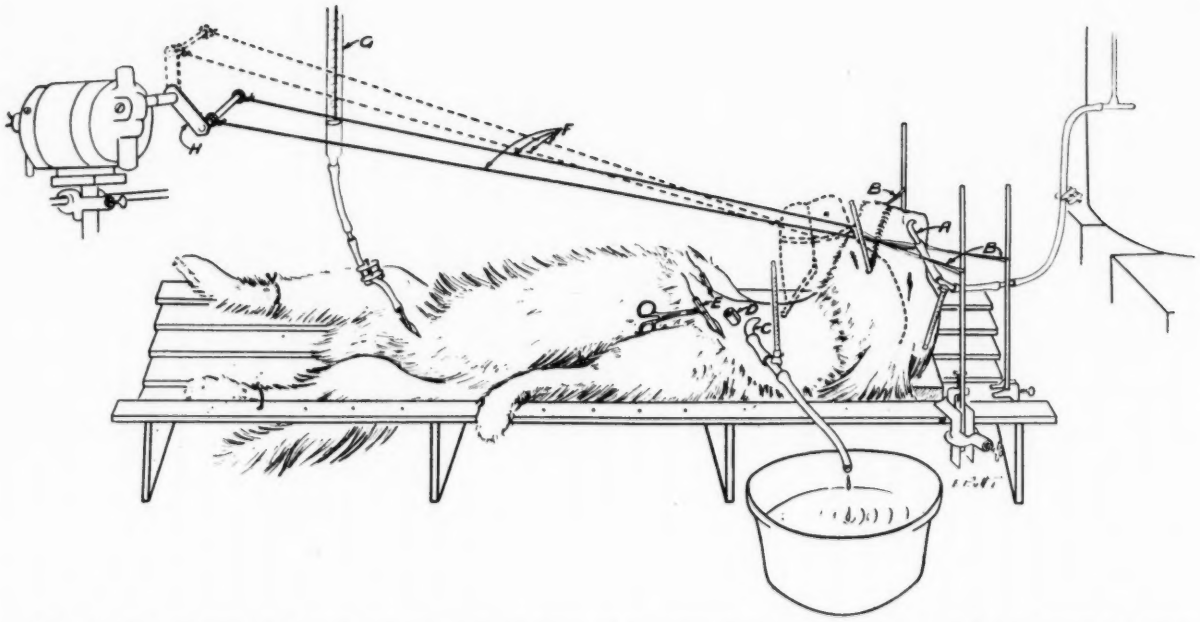

Fig. 1. Diagram of apparatus for passive motion of the head and perfusion of the nasopharynx. *A*, inflow perfusion tube from constant temperature reservoir to nostril; *B*, rubber bands; *C*, outflow perfusion tube tied into trachea; *D*, tracheal cannula; *E*, cannulae in cervical lymphatics; *F*, twine attaching snout to crank; *G*, burette attached to cannula in femoral vein; *H*, electrically driven crank. When the motor rotates the crank, *H*, the head is flexed and the rubber bands, *B*, return it to the usual prone position. This slight passive motion results in a constant flow of cervical lymph and produces ideal conditions for studying the absorption of various substances from the nasopharynx.

palate, to record the temperature of the mucous membrane. The lips were sewed together, thus preventing the escape of perfusion fluid via the mouth.

Since these procedures hindered the regulation of body temperature by preventing heat loss from the mouth, it was found necessary to direct an electric fan on the surface of the body in order to prevent a rise in body temperature. In some cases, it was necessary to supplement the fan with wet cloths or ice packs. The rectal temperature was observed frequently.

Lymph flowing into the two cannulae, during a definite length of time, was removed and placed in small weighed test tubes. The tubes were then reweighed; the percentage of lymph protein determined refractometrically; and the milligrams of lymph and of protein appearing per

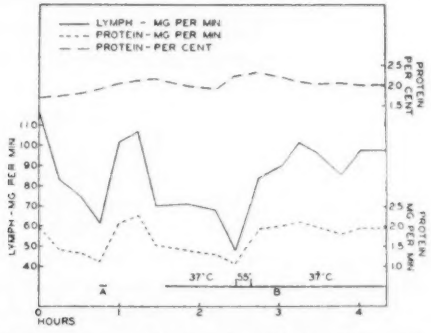

Fig. 2. Chart illustrating sensitivity of method. The upper curve shows the percentage of protein in the pooled lymph from both cervical ducts of a dog. In the lower curves are charted the total amounts of lymph and of lymph protein collected per minute. During *A*, the external jugular veins were clamped for six minutes; during *B*, the nasopharynx was perfused with Ringer's solution at the temperatures indicated. Note the changes in cervical lymph flow and in protein content caused by the increase in venous pressure and by the local application of heat (55°C.).

minute in the cervical lymph thus calculated. By this quantitative method, small changes in lymph flow can be detected readily.

RESULTS. 1. *Sensitivity of the method.* Three procedures were used in order to determine the sensitivity of the method in detecting changes in lymph flow. These procedures were: *a*, the rapid intravenous injection of a large quantity (500 cc. in five minutes) of Ringer's solution; *b*, an increase in venous pressure by temporarily clamping the external jugular veins; and *c*, the application of heat by nasopharyngeal perfusion with Ringer's solution at 55°C. Each of these procedures is known to cause an increase in lymph formation as a result of greater capillary filtration (Drinker and Field, 1933). With each manoeuver there was an immediate rise in the milligrams of lymph appearing in the cannulae per minute. Figure 2,

taken from a single experiment in which two of the three procedures were employed, illustrates this fact. The increase in lymph output which occurred following clamping of the external jugular veins for six minutes was transitory, due to a temporary rise in capillary pressure. Perfusion of Ringer's solution at 55°C. through the nasopharynx caused a more prolonged effect due to active hyperemia and to damage of the capillaries as a result of the heat.

2. *Normal cervical lymph flow and protein content.* In every experiment, passive motion of the head caused a flow of protein-containing lymph

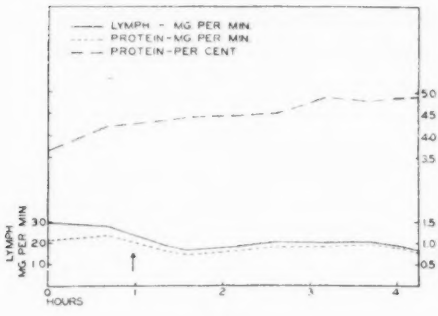

Fig. 3

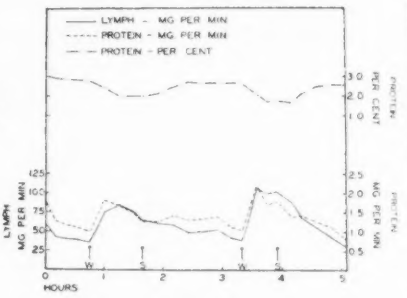

Fig. 4

Fig. 3. Chart illustrating the relatively little effect of nasopharyngeal perfusion with Ringer's solution on the flow and protein content of the cervical lymph of a dog. The upper curve shows the percentage of protein in the pooled lymph from both cervical ducts. In the lower curves are charted the total amounts of lymph and of lymph protein collected per minute. Perfusion with Ringer's solution at 37°C. was begun at the point marked by an arrow and continued throughout the experiment.

Fig. 4. Chart illustrating the changes produced in the flow and protein content of the cervical lymph of a dog as a result of nasopharyngeal perfusion with distilled water. The upper curve shows the percentage of protein in the pooled lymph from both cervical ducts. In the lower curves are charted the total amounts of lymph and of lymph protein collected per minute. At arrows, *W*, the nasopharynx was irrigated with distilled water; at arrows, *S*, the distilled water was replaced with Ringer's solution.

from the cannulated cervical lymphatics. Some animals produced more than others, but in no case was it impossible to obtain lymph. Massage was never necessary. The initial amounts of lymph and protein were often high, due to the mechanical effects of cannulation, etc. As conditions became more constant, there was a rather quick drop to a lower level. In the case of lymph, this level of amount averaged 53.3 mgm. per minute. For lymph protein, the average was 1.49 mgm. per minute.

3. *Lymphatic absorption of Ringer's solution from the nasopharynx.* The apparent ease of lymphatic absorption from the nasopharynx has already

been remarked upon. Lymph from this region draining into the cervical lymphatics could be derived from two sources: namely, *a*, capillary filtration of blood plasma; or *b*, direct lymphatic absorption of fluid from the nasopharynx. If the latter occurred to any great extent, nasopharyngeal perfusion of Ringer's solution should cause an increase in the amount of cervical lymph, with a decrease in its protein concentration, due to dilution of tissue fluid. Contrary to expectations these changes did not occur. Figure 3 is a chart of the results obtained from a typical experiment. Cervical lymph was collected during a control period. Perfusion of the nasopharynx with Ringer's solution at 37°C. was begun at the point marked by an arrow, and was continued until the conclusion of the experiment three hours and twenty minutes later. Perfusion caused no significant changes in the milligrams of lymph obtained per minute, nor in the milligrams of lymph protein per minute. The lymph:protein ratio decreased during the course of the experiment from 27:1 to 20:1, while the percentage of protein in each sample increased. These facts indicate that the lymph was becoming more concentrated, rather than less concentrated. When a 5 per cent solution of T-1824 was placed in the nasopharynx, the dye appeared in the lymph in one hour and twenty minutes, showing that some lymphatic absorption was taking place. The same results were obtained in many similar experiments. Since quantitative measurements were employed, however, it is evident that the normal lymphatic absorption of isotonic fluid from the nasopharynx must be extremely small.

4. *Lymphatic absorption of distilled water from the nasopharynx.* In contrast to the results obtained by perfusion with Ringer's solution, irrigation of the nasopharynx with distilled water caused an immediate and marked increase in the flow of cervical lymph. This is illustrated in figure 4. At the arrows, *W*, the nasopharynx was perfused with distilled water. When a definite change in the minute output of cervical lymph occurred, the distilled water was replaced with Ringer's solution as indicated by arrows, *S*. During the periods of distilled water perfusion, the amounts (milligrams per minute) of lymph and of lymph protein collected showed more than a two-fold increase over the amounts obtained during the preceding control periods. Although the lymph protein in milligrams per minute increased, the percentage of protein decreased. This indicated that the lymph had been diluted by direct absorption of water from the nasopharynx. When distilled water was replaced by Ringer's solution, the lymph flow and the lymph protein, as well as the percentage of protein, returned to their respective control levels.

Prolonged perfusion with distilled water, alternating with Ringer's solution, produced extensive damage to the nasopharyngeal epithelium. Histological sections of mucous membrane, removed at the termination of this experiment, showed partial or complete destruction of the epithelial

layer in many regions. In some places, only the outer cells had disintegrated, with many of the remaining cells appearing swollen. Considering the extent of the damage, it is rather surprising that the mucous membrane was so effectively impermeable to Ringer's solution during the last stage of the experiment.

This last observation, together with the fact that the initial application of distilled water caused an immediate increase in the cervical lymph flow, before extensive damage could have occurred, indicates that the differences in the amount of lymphatic absorption accompanying the use of the two solutions may be explained on the basis of differences in osmotic pressure relations. The distinctly hypotonic distilled water was readily absorbed, due to the combined osmotic effects of the salts and of the proteins in the body fluids. With Ringer's solution, on the other hand, which has the same salt content as the body fluids but lacks colloids, the only effective osmotic pressure was exerted by the body-fluid proteins, and since this pressure is relatively small, very little, if any, fluid was absorbed.

The experiments of Yoffey and Drinker and their co-workers on the lymphatic absorption of specific substances do not necessarily disagree with the present findings, since the substances reported by these investigators were detected in lymph in relatively small concentrations. Colloidal dyes, egg albumin, serum albumin and type III pneumococci passed through the nasopharyngeal mucosa and appeared in the cervical lymph in readily detectable concentrations; but, on the whole, it seems highly probable that the actual amount of absorption from the nasopharynx during normal physiological conditions cannot be as great as suggested by these earlier experiments.

SUMMARY

A new quantitative method for the production of an even flow of cervical lymph in anesthetized animals is described. The method involves regular passive motion of the head; is free of the complications introduced by the massage, heretofore usually necessary in order to secure lymph; and may be combined with perfusion of the nasopharynx in the study of lymphatic absorption from this area.

Experiments are reported showing that lymphatic absorption from the nasopharynx during nasopharyngeal perfusion with Ringer's solution is too small to be measured quantitatively. Perfusion of the nasopharynx with distilled water results in extensive lymphatic absorption of fluid with consequent dilution of cervical lymph,— a result believed to be due to the osmotic pressure of body-fluid salts and proteins acting on the hypotonic distilled water. During normal physiological conditions, the lymphatic absorption of fluid from the nasopharynx probably takes place to a very slight degree only.

The writer wishes to express her appreciation to Dr. Cecil K. Drinker for the suggestion of this work, and for the help he has given during the experiments.

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INHIBITION OF GASTRIC MOTILITY ASSOCIATED WITH THE PRESENCE OF PRODUCTS OF PROTEIN HYDROLYSIS IN THE UPPER SMALL INTESTINE

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The presence in the upper small intestine of HCl, (Boldyreff, 1904) fat, (Edelmann, 1906) or some of the carbohydrates (Quigley, Johnson and Solomon, 1929; Quigley and Hallaran, 1932) is associated with inhibition of gastric motor activity. Brunemeier and Carlson (1914) observed inhibition of hunger contractions when 10 per cent Witte's peptone in 0.2 per cent HCl was placed in the duodenum. We have reported that neutral solutions of various commercial "peptones"[1] cause inhibition of gastric peristalsis when they are placed in the duodenum (Thomas and Crider, 1936; Crider and Thomas, 1938).

The present study was undertaken to extend our observations and to learn whether similarly active products are formed in the ordinary course of protein digestion in the living animal. We have also compared the action of the different classes of products of protein digestion (proteoses, peptones, amino acids) and have attempted to make an analysis of the mechanism involved in the gastro-inhibitory effect of these substances.

METHODS. All the experiments were performed on dogs provided with cannulated gastric and duodenal fistulas as described previously (Thomas, Crider and Mogan, 1934). Nine animals were used, each of which was kept in the laboratory long enough to overcome its fear of the experimental procedures and to permit of a careful study of its responses on an individual basis.

That part of the apparatus which was placed within the stomach and intestine is shown in figure 1. Antral peristalsis was recorded by the balloon method using a bromoform manometer and kymograph. In the early experiments material to be placed in the duodenum was injected through the upper (drain) tube and delivered into the first part of the duo-

[1] The term "peptone" is used by manufacturers to designate any mixture of the products of incomplete protein digestion. When we have used the term in this sense, except as part of a trade name, we have enclosed it in quotation marks; otherwise it refers to products of protein digestion, other than the amino acids, which are soluble in saturated ammonium sulphate solution.

denum. Later we found that more consistent results were obtained when the material was injected through the lower (injection) tube into the lower duodenum or upper jejunum and we therefore adopted the practice of making all injections into this region.

The animals were fed once daily and, when used, were brought to the laboratory before being fed. First, the tubes and balloon were inserted and the activity of the empty stomach recorded and the effect of the material under investigation on this type of motility was observed. The animal was then fed a meal consisting of 300 to 500 grams of raw lean beef and the experiments were repeated while digestion was in progress. Only quantitative differences in the reactions of the full and empty stomach

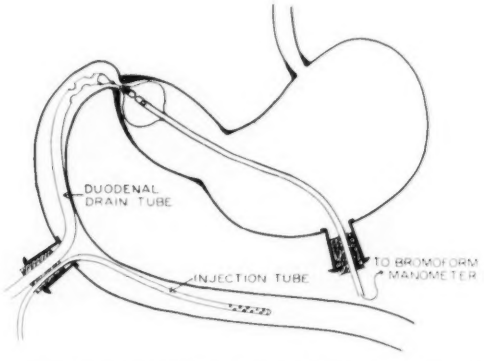

Fig. 1. Diagram showing the position of the tubes and balloon within the stomach and intestine and the approximate position of the gastric and duodenal cannulas. The perforated part of the injection tube began about 8 inches beyond the duodenal cannula; the cannula was between 6 and 8 inches from the pylorus. A Rehfuss tip (not illustrated) was attached to the inner end of the injection tube.

were observed, hence it is not necessary in reporting our results to distinguish between hunger activity and digestive peristalsis.

After the animal was fed the upper duodenal (drain) tube was left open to prevent chyme from passing down the intestine. This procedure had the effect of keeping the intestinal receptor mechanism in a state of high sensitivity and greatly facilitated the demonstration of gastro-inhibitory effects. It also causes a progressive increase in the force of gastric peristalsis (Thomas, Crider and Mogan, 1934) which must be controlled by frequent injections of inhibitory material into the intestine or the animal will eventually show signs of distress and may vomit.

The materials to be studied were prepared in neutral, isotonic solution. The pH values were determined colorimetrically, or electrometrically with a quinhydrone electrode. Osmotic pressures were estimated by the

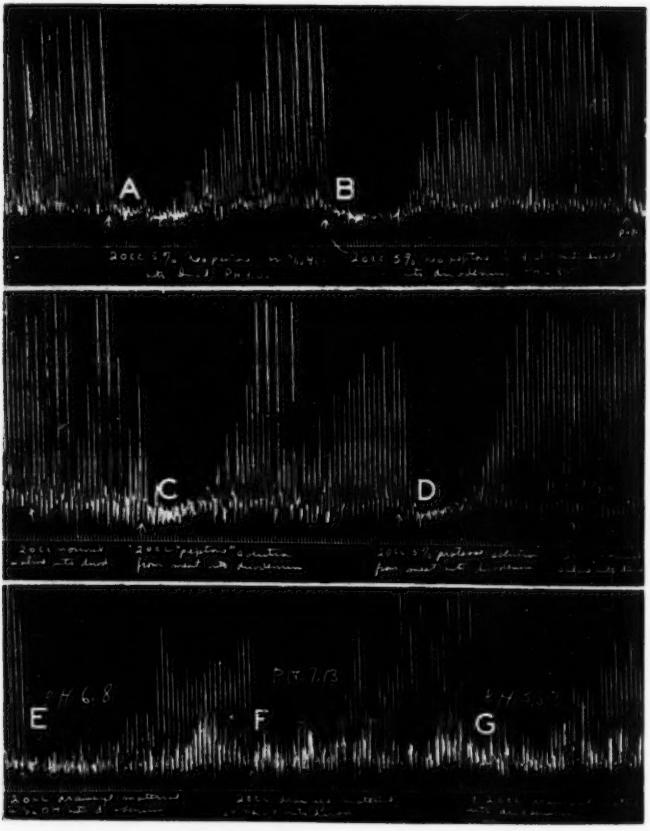

Fig. 2. Effects on antral peristalsis of placing various products of protein hydrolysis in the upper small intestine.

Top record: *A*, 20 cc. of a 5 per cent neo-peptone solution in N/10 HCl (pH 3.05); *B*, 20 cc. of the same solution after neutralization with NaOH to pH 6.85.

Middle record: effects of the different fractions of the intestinal contents collected after a previous feeding of raw, lean beef; *C*, 20 cc. of the proteose-free fraction; *D*, 20 cc. of a 5 per cent solution of the proteose fraction. The two solutions had the same percentage of total nitrogen.

Bottom record: effects of reinjecting 20 cc. portions of the total intestinal contents collected earlier in the experiment; *E* and *F*, neutralized with NaOH to pH 6.8 and 7.13 respectively; *G*, not neutralized (pH 3.53).

All the records were obtained within 2 hours after feeding. Time is recorded in 10 second intervals.

freezing point method and sodium chloride added when necessary; the material was considered satisfactorily "isotonic" if the lowering of the

freezing point was between 0.5 and 0.6°C. The concentration of protein material, when not known, was calculated on the basis of total nitrogen. The nitrogen determinations were made for us in the Jefferson Hospital laboratory under the direction of Dr. Abraham Cantarow.

The various materials used and the methods of preparing them will be described under the head of results.

RESULTS. *Commercial "peptones."* The following commercial "peptones" were studied: Witte's peptone, bacto-peptone, neo-peptone, proteose-peptone, and bacto-protone. Each of these substances, when placed in the upper small intestine in adequate concentration, caused inhibition of gastric motility whether the stomach was full or empty. The response to 20 cc. of a 5 per cent solution of neo-peptone, which is typical of the group, is illustrated in figure 2, *B*. Five per cent solutions were generally used but under favorable conditions definite inhibition may be obtained with dilutions as low as 0.5 per cent (Witte's peptone).

No differences in the type of response to the different "peptones" were observed and, with the exception of the first two mentioned, they gave results that were quite similar in degree. Witte's peptone caused greater and more prolonged inhibition of the stomach and bacto-peptone caused less inhibition than any of the others. This difference was observed in our earliest experiments. It happens also that Witte's peptone contains a higher, and bacto-peptone a lower percentage of proteose than the other commercial "peptones" investigated. This coincidence misled us into stating the opinion that the gastric inhibition was probably caused mainly by the proteoses. We are now convinced that the proteoses in general are no more potent as stimuli to the inhibitory mechanism than are other products of protein digestion. We have no information which sheds any light on the difference in effectiveness of these "peptones."

The onset, degree and duration of gastric inhibition following the injection of "peptone" solutions into the duodenum resemble closely the effects of HCl under similar circumstances. In this respect, 5 per cent Witte's peptone in neutral solution is approximately equivalent to N/10 HCl. Considerable variations in the degree of inhibition caused by identical "peptone" solutions in the same animal occur from time to time and these are paralleled by similar variations in the response to HCl.

The effect of "peptone" on gastric emptying. The gastric emptying time for 300 cc. of a 5 per cent solution of Witte's peptone was measured in six experiments on each of two animals and compared with the emptying time for an equal amount of normal saline. The method was the same as that used in a previous study (Crider and Thomas, 1937). The "peptone" solutions took, on the average, approximately 3 times as long (55 and 78 min. in the two animals respectively) to leave the stomach as the saline (19 and 24 min.). The "peptone" emptying times ranged from 30 to 80

minutes in one animal and from 70 to 100 minutes in the other, smaller, animal. Saline emptying times ranged from 10 to 30 minutes.

The longer emptying time for the "peptone" solutions is not all attributable to inhibition of gastric motility. "Peptone" also excites gastric secretion, increasing both the volume and acidity of the gastric contents. However, slower emptying of the "peptone" solutions was evident even during the first ten minutes after the stomach was filled, and before the effect on gastric secretion could have been important. The average volumes remaining in the stomach at the end of 10 minutes were 191 and 209 cc. of "peptone" solution and 112 and 111 cc. of saline in the two animals respectively.

Products prepared from the intestinal contents. Experiments were undertaken to determine whether the products of protein digestion in the normal animal include inhibitory substances similar to those found in commercial "peptones." The animals were fed raw lean beef and the total intestinal contents collected. The collected material was boiled and filtered to remove coagulable protein and the proteose fraction precipitated by saturation with ammonium sulphate. After one or more reprecipitations the proteose precipitate was extracted several times with ether to remove fat, carefully freed of reagents and prepared in neutral, isotonic solution. When these preparations were placed in the intestine gastric inhibition occurred similar to that caused by the commercial "peptones" (fig. 2, *D*).

The filtrate containing the residue of the intestinal contents after removing the proteose was treated to remove reagents and ammonia and concentrated (or diluted) to the same total nitrogen content as the proteose solution with which it was compared. The composition of such a preparation is uncertain but it is known to contain peptones and amino acids. Gastric inhibition equal to or greater than that caused by the proteose solutions occurred when this material was placed in the intestine (fig. 2, *C*). Generally the latent period was a little shorter than that following the injection of proteose.

The ammonium sulphate was removed from the preparations described by boiling with barium carbonate. This treatment probably caused some degree of further hydrolysis, and, possibly, other changes. In the hope of obtaining evidence free from such objections a study was made of the untreated intestinal contents during meat digestion. Dogs were fed the usual meal of raw beef free from visible fat. The contents of the first part of the duodenum were drained to the outside and collected. Measured amounts (usually 20 cc.) of the drainage material were injected at intervals via the lower (injection) tube. Such injections caused gastric inhibition, generally greater in degree than that following equal amounts of 5 per cent proteose solutions (fig. 2, *G*).

Since the material drained from the duodenum was acid in reaction the following experiments were performed to determine to what extent the acidity was responsible for the inhibition. After a suitable quantity of intestinal contents was collected it was divided into approximately equal fractions, one of which was neutralized with concentrated sodium hydroxide solution and the other left unchanged. Customarily the pH of both fractions was measured with a quinhydrone electrode. Injection into the duodenum of equal quantities of the two fractions caused approximately equal grades of inhibition of the stomach (fig. 2, bottom record), except when the untreated material was more acid than pH 3.0. Such a degree of acidity is rare in the duodenal contents but when it was present the untreated, acid, material caused slightly (but definitely) more gastric inhibition than the same material after neutralization.

Similar observations were made with commercial "peptone" solutions. Acidification of the solutions with HCl did not increase the inhibitory action (fig. 2, *A* and *B*) unless the solution was made more acid than pH 3.0.

Primary and secondary proteoses and peptones. The precipitates obtained on half saturation and on subsequent complete saturation of a Witte's peptone solution with ammonium sulphate were prepared separately as described above. The precipitate obtained on complete saturation of a neo-peptone solution with ammonium sulphate and the residue remaining in solution were prepared in a similar manner. Each of these preparations caused gastric inhibition when placed in the intestine. The primary and secondary proteoses prepared from Witte's peptone exhibited the strong inhibitory effect characteristic of the original mixture. There was little if any difference, gram for gram, in the activity of the total proteose prepared from neo-peptone, the residue after precipitation of the proteose, and the original mixture. In general, the latent periods for inhibition following the injection of proteose preparations were slightly longer than those for the other substances.

The proteose solutions may reasonably be assumed to have been free from significant contamination with other products of protein digestion and the experiments, in our opinion, provide a satisfactory demonstration of the independent inhibitory effect of these substances on gastric peristalsis. The residue of the neo-peptone after removal of the proteose fraction should consist (according to the manufacturer's analysis) of about 88 per cent true peptones and 12 per cent amino acids. It is probable, therefore, that the inhibitory effect of this mixture was due mainly to the true peptones. The amino acid content, unless greatly increased during the manipulations, would be sufficient to account for only a small part of the observed inhibitory effect of the preparation.

Protein digests prepared in vitro.[2] The following digests of casein were prepared and their inhibitory action on gastric peristalsis compared:

Pepsin digest (24 hours in acid solution at 40 degrees)

Trypsin digest (6 hours in neutral solution at 40 degrees)

Pepsin-trypsin digest (24 hours in acid solution with pepsin and three weeks in neutral solution with trypsin at 40 degrees)

Acid digest (12 hours in the autoclave at 20 lbs. pressure with 25 per cent sulphuric acid)

Sulphuric acid was used to acidify the material in preparing the pepsin digests because it is easily removed from solution. For a similar reason the trypsin digests were buffered with barium carbonate.

The proteose fractions of the pepsin and trypsin digests and the residue of the trypsin digest after precipitation of the proteose were also studied. As in the other experiments all the preparations were freed of reagents and ammonia and prepared in neutral, isotonic solutions of known concentration.

The object of these experiments, besides adding to the number of products of protein digestion investigated, was to see if any differences in inhibitory effect could be detected in the products produced by different hydrolytic agents and to determine whether the property of causing gastric inhibition was affected by the degree of hydrolysis.

Each of the preparations caused gastric inhibition when placed in the intestine and all were about equally effective. Twenty cubic centimeters of a solution containing 1 per cent of protein hydrolysate proved to be about the minimal dose for reliable results. There was evident a definite difference in the latent periods, those following the injection of proteose solutions being longer than those following the injection of the products of more complete digestion. We are inclined to attribute this fact to the lesser mobility of the larger molecules. Since even the longer latent periods seldom exceeded one minute, it does not seem probable that significant digestion of the proteoses occurred within the intestine before the appearance of gastric inhibition.

Amino acids.[2] A study of the various amino acids, separately and in groups, has been undertaken. The results have proved to be so complicated that it seems best to reserve a complete description for a later communication. At present we are prepared to report that when the amino acid mixture obtained from the complete hydrolysis of casein is placed in the intestine it causes gastric inhibition comparable in degree to that caused by proteoses and peptones. Some of the individual amino acids appear to be inert in this particular and others more active than the products of incomplete digestion. Of the amino acids capable of causing

[2] Because of increasing executive responsibilities Doctor Crider was unable to participate in this part of the investigation.

gastric inhibition in neutral solution, the monoaminomonocarboxylic acids are the more potent.

Native protein. The only native protein material investigated was egg white. This substance was tried in neutral solution and in acid solutions of various degrees of acidity. The presence of neutral egg white in the intestine had no effect on gastric motility. In acid solution it had no more effect than an equivalent amount of inorganic buffer.

A commercial preparation of gastric mucin caused moderate inhibition of gastric peristalsis when perfused through the lumen of the duodenum and upper jejunum but the possibility that some products of protein hydrolysis were present in the material could not be excluded.

Latent period. In all the experiments in which gastric inhibition occurred it began promptly after the beginning of the injection of the inhibitory substance into the intestine and was frequently evident before the injection was completed. In experiments (fig. 3, top record) in which alternate injections of normal saline and "peptone" solutions were made from burettes placed side by side and filled to the same level, the saline injections were generally without effect, proving that the initial inhibition was not a response to mechanical distention of the intestine. Under these conditions the minimal latent period for inhibition following the beginning of the injection of a potent "peptone" solution was approximately equal to the interval between gastric peristaltic waves (about 15 seconds in the dog).

The effects of vagotomy. Experiments with Witte's peptone and neopeptone were performed on two animals after both vagi had been cut in the neck, and after the lapse of a sufficient time, following the vagotomy, to permit of the recovery of gastric peristalsis. No unequivocal inhibitory effects were obtained after vagotomy (fig. 3, middle and bottom records). In a few experiments there was a slight reduction in the amplitude of gastric peristalsis (fig. 3, middle record) following the injection of a "peptone" solution into the duodenum but such results could not be repeated consistently and were, possibly, coincidental.

Attempts were made to stimulate the humoral inhibitory mechanism with "peptone" solutions following vagotomy by the repeated injection of large amounts of material into the intestine but all the results were negative. If, as sometimes happened, moderate inhibition followed the first injection, it promptly disappeared and subsequent injections were without inhibitory effect. Occasionally gastric activity increased following repeated injections of "peptone" into the intestine but these results, too, were inconstant.

Discussion. The experiments described we think are adequate to establish the fact that most of the products of protein digestion are capable of initiating inhibition of gastric motility when they are present in the

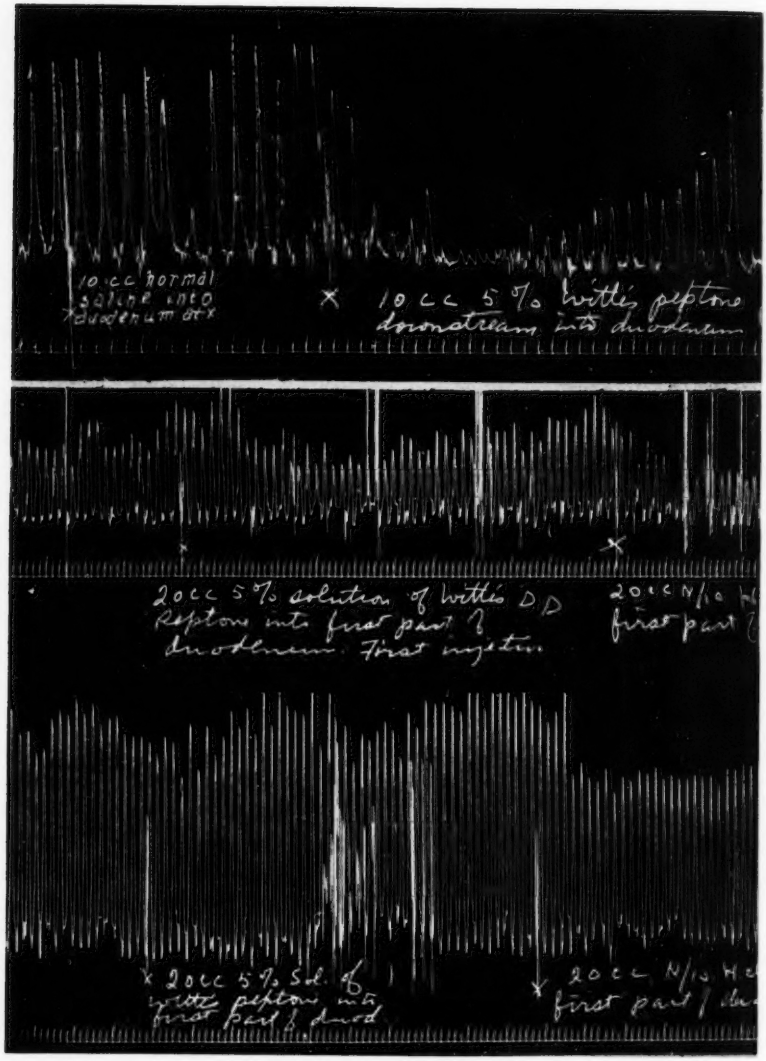

Fig. 3. Records of experiments designed to determine the mechanism of the gastric inhibition caused by "peptone."

Top record: Measurement of the latent period; the line marked *X* was made by squeezing a bulb on a side branch of the manometer tube at the moment the solution began flowing into the duodenum.

Middle and bottom records illustrate results obtained after double cervical vagotomy. The middle record is the type described in the text as indicating the possibility of some degree of residual inhibition; the bottom record is the more characteristic. Toward the end of the middle and bottom records the effect of 20 cc. of N/10 HCl may be seen.

Time in 10 second intervals.

intestine. They indicate quite clearly that some such inhibitory substances are present in the intestinal contents in effective concentration during the digestion of a protein meal. The results may reasonably be interpreted further as indicating that each of the recognized classes of hydrolytic products (proteoses, peptones and amino acids) includes some gastro-inhibitory substances.

The fact that "peptone" preparations having a strong gastro-inhibitory effect in the intact animal have no such effect in the same animal after double vagotomy indicates that the gastric inhibition is the result of a reflex involving the vagus nerves. Any interpretation which would attribute the inhibition to a humoral mechanism is, we think, excluded in this particular instance by the brevity of the latent period, which is sometimes less than the circulation time.

The results are given added significance by the previous observations that fat and carbohydrate in the intestine inhibit gastric peristalsis and delay gastric emptying. It is now evident that each of the major foodstuffs, either in its natural state or after partial digestion, is capable of exerting a regulatory influence from the intestine on gastric motility. In the order of potency in this regard fats come first, products of protein digestion second and carbohydrates third. It is probably significant that, as Cannon observed, the gastric emptying times for the three types of food decrease in this same order. These relationships suggest that the rate at which the stomach empties each type of food is in large part determined by the potency of the gastro-inhibitory effect of the material reaching the intestine. It is suggested that a mixed meal is emptied from the stomach at such a rate as to maintain at, or just above, threshold value the combined reflex and humoral gastro-inhibitory effects of all the substances making up the intestinal contents.

SUMMARY. 1. The list of substances (fat, carbohydrate, HCl, etc.) capable of causing gastric inhibition when placed in the upper small intestine has been extended to include various products of protein digestion.

2. Among the numerous materials investigated and found to cause gastric inhibition when placed in the intestine in neutral solution were: commercial peptones, the intestinal contents obtained from dogs during meat digestion, various enzyme and acid digests of casein prepared in vitro, the separated proteose and proteose-free fractions of several of the above, various individual amino acids and amino acid mixtures. Some of the individual amino acids were ineffective.

3. The gastro-inhibitory effects of all the substances studied were qualitatively similar and resembled the effect of HCl. Some quantitative differences in activity were observed but not explained; they did not appear to be correlated with the hydrolytic agent used nor with the degree of hydrolysis.

4. The minimal latent period for gastric inhibition in these experiments was about 15 seconds.

5. No gastro-inhibitory effects were observed after double cervical vagotomy, in experiments limited to two animals and two of the commercial peptones.

CONCLUSION

The products of protein hydrolysis cause inhibition of gastric motility by acting in the intestine as stimuli for the enterogastric reflex.

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THE FIFTH STAGE OF NEUROMUSCULAR TRANSMISSION

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While repeating and controlling some experiments made by Bowditch in 1890 upon continuous tetanization of a motor nerve after injections of curare, the observation was made (Luco and Rosenblueth, 1939) that some minutes to hours after total decurarization muscles which had not contracted—first because of curare, later because of transmission-fatigue—gradually began to develop some tension until a fairly high plateau was reached. This tension could then be maintained for hours without any evidence of further fatigue. Bowditch had observed this late development of tension but had attributed it to the disappearance of curare. Since in our experiments total decurarization usually took place before the tension began to develop we inferred that the late responses were not related to the decurarization. This inference led to the study of muscular contraction upon prolonged continuous stimulation of motor nerves at frequencies which would lead to transmission-fatigue. The present report deals with this study. Its results justify the inference that a late development of tension succeeds complete fatigue of transmission whether or not curare is previously injected.

METHOD. Cats were used, either anesthetized with dial (Ciba, 0.7 to 0.75 cc. per kgm. intraperitoneally) or decerebrated during a brief ether induction. The muscles studied were those attaching to the Achilles tendon. Sometimes all these muscles were made to record together but, more frequently, the soleus was separated from gastrocnemius-plantaris, and either of these muscles recorded independently. The legs were fixed by drills inserted into the tibiae. The contractions of the muscles were recorded on a kymograph by myographic levers of the 3rd type, pulling against one or more heavy rubber bands. Upward excursions denote contraction. The magnification was from 8- to 15-fold. The records indicate mainly changes of tension.

Shielded silver electrodes were applied to the popliteal nerves, after severance of the sciatic, for either stimulation or recording purposes. Care was taken to preserve intact the blood supply of the nerves. The

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electric responses of the muscles were led to a capacity-coupled amplifier from platinum needles inserted, usually, one in the tendon, the other in the belly of the muscle. The amplified signals were photographed from a cathode-ray oscillograph.

The stimuli employed were either condenser discharges with frequency regulated by a thyratron, or short rectangular pulses from a multivibrator circuit. Such stimuli were not delivered directly to the nerves, but sent into the primary coils of transformers. The diphasic output of the secondary coils was found satisfactory for the prolonged periods of stimulation required. The polarity of the shocks was used which yielded better responses upon submaximal stimulation. The intensity was then increased till slightly supramaximal. This supramaximality was tested several times during each experiment. It was seldom necessary to increase the intensity of the stimuli in the course of an observation.

RESULTS. A. *The 5th stage of neuromuscular transmission.* When a motor nerve is stimulated at a sufficiently high frequency, the muscle responds with a complex sequence of changes of tension. The initial rise is promptly followed by a drop; this in turn is succeeded within a few seconds by another rise of tension; and then, commonly with additional irregularities, the slow decline interpreted as "fatigue" takes place. The initial $+ \rightarrow - \rightarrow +$ sequence may be denoted as the *1*st, *2*nd and *3*rd stages of neuromuscular transmission. This study is not concerned with these three stages (cf. Rosenblueth and Morison, 1937*a*, for a discussion of the *2*nd and *3*rd). Indeed, the frequencies of stimulation employed here were too low for the appearance of the *2*nd phase. The *1*st and *3*rd were thus merged together, and, neglecting, because of insufficient knowledge, the intermediate irregularities mentioned above (see fig. 6), they were followed by a slow decline of tension ("fatigue"), which will be spoken of as the *4*th stage of transmission. If the stimulation is continued a late development of tension appears, which will be referred to as the *5*th stage.

For instance, if the motor nerve to either gastrocnemius-plantaris or soleus is stimulated at a frequency of 60 per sec., the initial development of tension subsides first more rapidly, then more slowly until, 12 to 60 min. after the beginning of stimulation very little or no measurable response is present. If the stimulation be continued the responses will remain minimal or absent for 1 to 2.5 hours, but eventually the tension will begin to increase. It then gradually rises in an S-shaped curve until 2 to 3 hours later a plateau is reached. The magnitude of the tension is then from 25 to 60 per cent of the highest tension developed at the beginning of stimulation (see table 2). This high tension can be sustained for a period of several hours. Thus, in one animal (fig. 10) a plateau of 58 per cent of the original maximal tension was sustained for a period of 3 hours, and in the subsequent 5 hours it only declined to 46 per cent. It is this late development

of tension succeeding the period of "fatigue" which we propose to call the 5th stage of transmission.

A characteristic feature of the 4th stage is the appearance of fine, irregular changes in the tension record (see figs. 8 and 13). These irregularities become more prominent during the 5th stage. The mechanogram shows fairly marked, arrhythmic ups and downs as long as stimulation is continued (figs. 1, 2, 6 and 8). It will be shown below that these irregularities are due to alternating activity of the muscle fibers.

At no time during the 5th stage is a contracture demonstrable. Typical muscle action-potentials are present (fig. 2). If the stimulus is momentarily stopped the muscle promptly relaxes to the level of the initial resting tension (fig. 9). This relaxation is slower than that of fresh muscles, as befits the fatigued condition, but it indicates that the contraction during

TABLE 1

Magnitude of the recorded A spike-potentials of peroneal nerves stimulated maximally for several hours

The responses are expressed as percentages of the original magnitude at the onset of stimulation. The times noted refer to the periods elapsed after this onset.

ANIMAL NUMBER	FREQUENCY PER SECOND	1 HOUR	2 HOURS	3 HOURS	4 HOURS	5 HOURS	6 HOURS
1	50	100	93	88			
2	60	106	108	106	106	100	
3	60	120	133	100	100		
4	60	100	94	94			
5	60	100	97	90	90		
6	60	100	100	94	88	79	73
7	60	100	97	89	79	69	
8	87	97	70	64	56	56	
9	97	71	55	47	43		

the 5th stage differs only quantitatively from that of the first stages of transmission.

B. *The lack of influence of dial anesthesia.* The majority of the experiments were carried out under dial anesthesia. In order to find out whether this anesthetic had any influence on the appearance and time course of the 4th and 5th stages, 4 decerebrate animals were tested. The results were entirely analogous to those obtained in the anesthetized cats.

C. *The lack of influence of the sympathetic nerve supply to the muscles.* Maximal stimulation of the popliteal nerve activates not only the motor fibers to the muscles, but also the sympathetic vasomotor elements which the nerve contains. A possible rôle of vasomotor phenomena in the appearance and development of the 4th and 5th stages was studied by stimulating the nerves in 4 animals 8 to 12 days after aseptic removal of the abdominal sympathetic chains from L1 to S2. In addition, the adrenal

glands were ligated at the start of the experiment in 2 of these animals. The results obtained were similar to those recorded without previous sympathectomy.

D. *The nerve action-potentials.* The electric responses of the nerves to stimulation over a period of hours were followed on the cathode-ray oscillograph and photographed periodically (about every 15 min.) in 9 cats. As

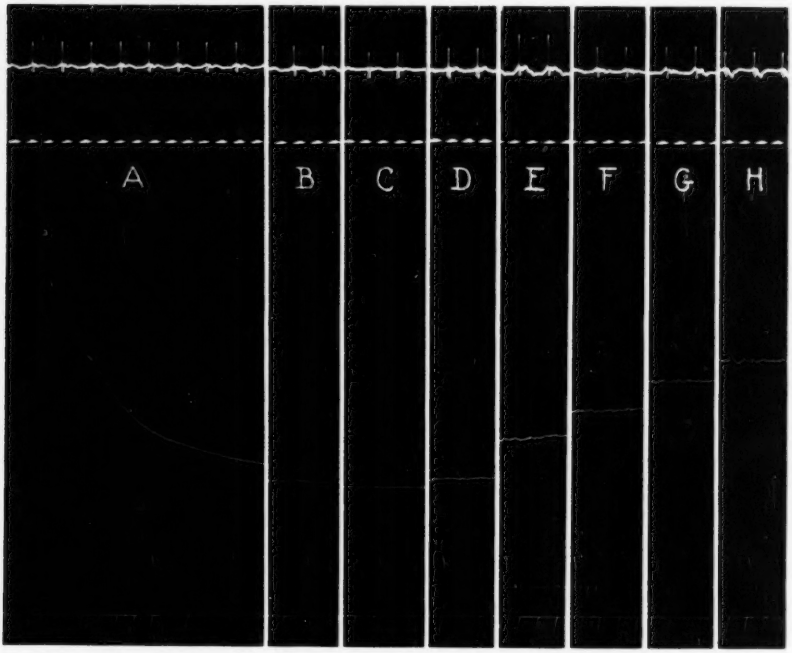

Fig. 1. Electric responses of popliteal nerve and mechanical response of gastrocnemius-plantaris muscle to continuous maximal stimulation at 60 per sec. The nerve spike-potentials were recorded during the corresponding kymograph record strips. A, beginning of stimulation. B to H, 30, 60, 110, 155, 180, 210 and 240 min. later, respectively. Time signals: mechanogram, 1 min.; electrogram, 10 msec. In this and in figures 2 and 12 the two upper signals in the kymograph records merely indicate times at which electrical records were taken; they are to be disregarded.

described under "Method" the lead-off electrodes were placed on an intact region of the nerve. The disadvantage of obtaining diphasic records was disregarded because of the advantages of preserving the blood supply of the nerves and of being able to follow concomitantly the muscular responses. The observations were concerned exclusively with the magnitude of the A spike-potentials.

The results are summarized in table 1. Up to frequencies of about 60 per sec. the electric responses did not decline significantly for about 2 hours. A progressive decrease of magnitude could take place thereafter (cats 1, 6 and 7). But even after 4 and 5 hours of continuous stimulation the spikes were full-sized in 2 cats (figs. 1 and 4).

With higher frequencies of stimulation (cats 8 and 9) a decline of the magnitude of the A spike-potentials took place earlier and was more

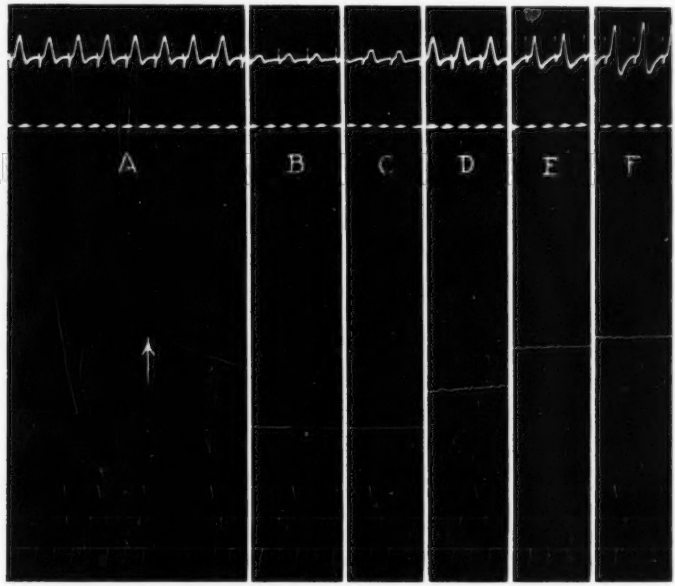

Fig. 2. Electric and mechanical responses of gastrocnemius-plantaris to prolonged indirect stimulation at 60 per sec. A, beginning of stimulation; the arrow indicates the time at which the corresponding electrogram was recorded; at the start of stimulation the muscle spike-potentials were 5 times larger. B-F, 45, 98, 135, 180 and 200 min. after the beginning of stimulation, respectively; the electric records were taken at the time of the corresponding kymograph record strips. Time signals: mechanogram, 1 min.; electrograms, 10 msec.

pronounced than with the slower frequencies (fig. 3). In no instance did even a marked (50 per cent or more) intensification of the stimuli result in an increase of the electric responses. In no case was there any evidence of alternation in the records. In no experiment, finally, did a slowing of the frequency of stimulation to $\frac{1}{2}$ or less produce any significant change in the responses.

The influence of rest-periods of 1 to 30 min. was tested in several of the animals after the spike-potentials had decreased in consequence of con-

tinuous stimulation for several hours. Only rarely was there a slight recovery toward the original magnitude after these rest-periods. Indeed, in several cases, the result of such interruptions was a further decrease, which became apparent when stimulation was renewed. A prompt marked treppe usually then reëstablished the magnitude prevalent before the rest-period.

Whether the recorded nerve impulses remained full-sized throughout the experiments (cats 2 and 3), or decreased in magnitude as stimulation was continued, in no case was there any correlation between the size of the

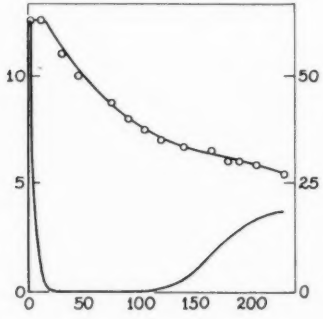

Fig. 3

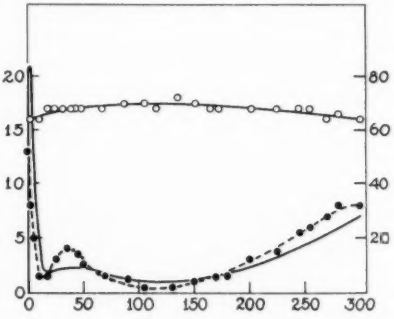

Fig. 4

Fig. 3. Responses of nerve and muscle to prolonged stimulation at 97 per sec. Lower continuous curve: mechanogram of gastrocnemius-plantaris, redrawn to scale from the original kymograph record. Upper curve (circles): magnitude of the diphasic A spike-potentials of the popliteal nerve. Abscissae: time in minutes after beginning of continuous stimulation. Ordinates: tension (right scale) and spike-potentials (left scale) in millimeters in the corresponding records.

Fig. 4. Responses of nerve and muscle to prolonged stimulation at 60 per sec. Lower continuous curve: mechanogram of soleus, redrawn from the kymograph record. Broken line (dots): average magnitude of the muscle spike-potentials. Upper curve (circles): magnitude of the A spike-potentials of the popliteal nerve. Abscissae: time in minutes after the beginning of continuous stimulation. Ordinates: tension (right scale) and spike-potentials (left scale) in millimeters in the corresponding records.

spike-potentials and the changes of tension of the muscle. Figures 1, 3 and 4 illustrate typically this independence.

E. *The muscle action-potentials.* The electric responses of the muscles were periodically (5 to 15 min.) recorded in 10 animals. The initial treppe of the electrograms and the marked decline which follows promptly at the start of stimulation are well known and need not be stressed here. Early in the period of "fatigue" (4th phase) slight irregular alternation invariably took place. This alternation in the electric responses coincided with the appearance of slight irregularities in the tension record. The initial

marked decline of the electrogram was usually followed by a slight but clear increase of the action-potentials, not necessarily correlated with any corresponding increase of tension (fig. 4). As the 4th stage progressed the electrograms decreased until a steady level was attained. Although in some of the experiments the muscular tension response was practically unmeasurable in some regions of the 4th stage, easily recognizable electric responses were invariably recorded throughout this period.

During the 5th stage the muscle spike-potentials increased until the average magnitude was 2 to 10 times greater than that which had prevailed during the 4th stage. This increase was usually parallel with the rise of tension (figs. 2 and 4). Exceptionally the increase of the electrogram lagged behind that of the mechanogram. Throughout the 5th stage alternation became very prominent in the electrograms. Although the records (fig. 5) clearly show this alternation, it was more striking when observed directly in the oscillograph. Changes of pattern and magnitude took place continuously without any orderly sequence. The increases

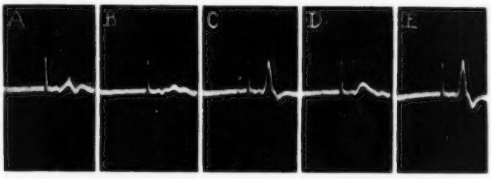

Fig. 5. Alternation in the electrograms of gastrocnemius-plantaris during the 5th stage. The 5 records reproduced were taken at about 1-sec. intervals. The first sharp diphasic excursion is the stimulus artifact.

appeared at the beginning or toward the end of the synchronized standing waves, showing marked differences in the latencies of the corresponding active units.

F. *The lack of influence of load.* By changing the number of rubber bands against which the muscles pulled, the load and the initial tension could be readily modified. In 3 cats the same muscle was hooked-up on the two sides and its nerve stimulated at the same frequency. The load was made, however, three times greater on one side than on the other.

In the 3 animals the time course of the 4th and 5th stages was the same on the two sides. The similarity of results comprised also approximately similar percentages of the initial maximal tension throughout the records for the two sides. It may be concluded that the load had no influence on the phenomena in these experimental conditions.

G. *The influence of frequency.* This influence was studied by recording from the same muscle on the two sides, with the same load, but stimulating the two nerves with different frequencies. In addition to these observa-

tions the results obtained from different animals were sufficiently consistent so that meaningful average figures could be determined. These average values supported the inferences drawn from the experiments performed upon a single animal.

With relatively low frequencies (e.g., 25 per sec. for soleus, or 30 per sec. for gastrocnemius-plantaris) even after several hours of stimulation the tension declined only slightly. It is therefore impossible to decide from

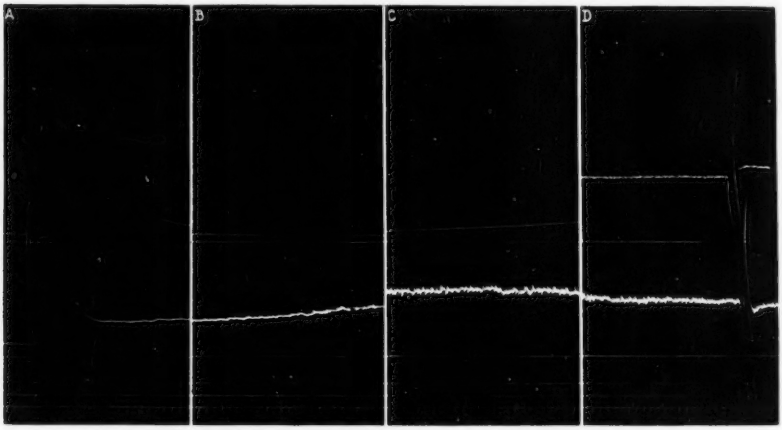

Fig. 6. Influence of frequency of stimulation on the 4th and 5th stages. Records from the soleus on both sides. Upper record: frequency 60 per sec. Lower record: frequency 95 per sec. Time signal: 1-min. intervals.

A. Beginning of continuous maximal stimulation.

B. Beginning of 5th stage for lower record. Time: 91 to 116 min. after start of stimulation.

C. Beginning of 5th stage for upper record. Time: 149 to 174 min. after start of stimulation.

D. Time: 269 to 294 min. after start of stimulation. The rest-period of 1 min. indicated by the signals was the first given in the observation.

In this and other records horizontal lines have been drawn immediately below the resting level of the muscles to facilitate the appreciation of the changes of tension.

the records whether some fibers were slowly falling into the 4th, and others simultaneously reaching the 5th stage, or whether the 5th stage did not appear at all at such slow frequencies.

The influence of the rate of stimulation was best seen with frequencies between 50 to 100 per sec. As the frequency increases within that range the decline into the 4th stage occurs more rapidly; the 5th stage, likewise, begins sooner. The tension of the late plateau of this stage is lower for higher than for less frequent rates of stimulation. In figure 6 is illustrated

a typical instance. The average figures given in table 2 equally support the previous statements.

With higher frequencies of stimulation (e.g., 150 to 300 per sec.) the separation of the 4th and 5th stages was not clear. Although the tension dropped more rapidly after the initial rise at the beginning of stimulation, throughout the 4th stage the tension remained higher than with lower frequencies (cf. fig. 6). Fairly soon (30 to 60 min.) the marked irregularities characteristic of the 5th stage made their appearance and usually a slight rise of tension was seen concomitantly. These results may be interpreted, in accord with the observations made with slower frequencies, by assuming that with high rates of stimulation the 5th stage begins for some fibers before all the elements have entered the 4th stage. The two

TABLE 2

Influence of frequency on the 4th and 5th stages

Times are expressed in minutes. The maximal tension during the 5th stage is measured as per cent of the maximal tension developed during the 1st stage at the onset of stimulation. In the column marked "number of animals" are indicated the number of observations of which the figures in the other columns are averages. The greatest deviations from the average are given in parentheses. The letters G.P. and S. denote the gastrocnemius-plantaris and soleus, respectively.

FREQUENCY	MUSCLE	NUMBER OF ANIMALS	TIME FOR MAXIMAL FATIGUE	TIME FOR BEGINNING OF 5TH STAGE	MAXIMAL TENSION DURING 5TH STAGE
60	G.P.	12	34 (15 to 60)	118 (75 to 190)	39 (29 to 48)
60	S.	9	18 (12 to 25)	152 (100 to 250)	40 (26 to 62)
80 to 100	G.P.	10	16 (3 to 40)	88 (45 to 130)	29 (21 to 37)
80 to 100	S.	4	9 (5 to 15)	68 (30 to 110)	30 (20 to 50)

phases thus become merged together and may not be easily recognized, but the 5th stage is present after 1 to 2 hours of stimulation.

If, after the 5th stage had been reached, the frequency was changed without interruption, the following results were recorded. A slowing resulted in an immediate fall of tension, succeeded shortly by an increase until a plateau was reached at a higher level than that which had prevailed at the higher frequency. Conversely, a change to a faster frequency caused an initial rise and a later fall of tension to a lower level than before the change (fig. 7). These results confirm the conclusion that the tension of the steady state during the 5th stage varies inversely with the frequency of stimulation.

H. *The differences between gastrocnemius-plantaris and soleus.* For the study of these differences the usual procedure was to record from one of the muscles on one side and from the other muscle on the opposite side,

while stimulating the nerves at the same frequency. Ten cats were thus studied. The conclusions of these experiments were further supported by the average values determined from the experiments made on different animals (table 2).

Fig. 7. Changes of frequency during the 5th stage. Record of the soleus muscle. The nerve had been stimulated at 60 per sec. for 5 hours, until the steady state of the 5th stage had appeared. The record begins 5 min. after the end of this period of stimulation. The successive signals denote: A, 60/sec. on; B, shift to 30/sec.; C, shift to 60/sec.; D, shift to 30/sec.; E, off. Time signal: 5-sec. intervals; the drum was speeded as shown by this signal.

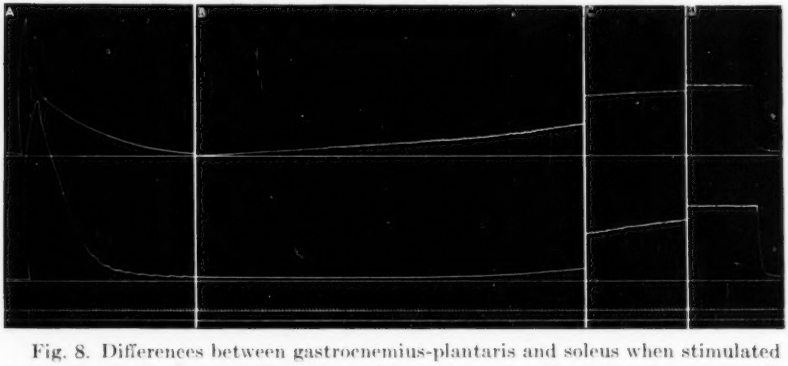

Fig. 8. Differences between gastrocnemius-plantaris and soleus when stimulated indirectly at 60 per sec. Upper record: gastrocnemius-plantaris. Lower record: soleus. Time signal: 30-sec. intervals.

A. Beginning of continuous stimulation. Length of record: 30 min.

B. Beginning of 5th stage, first for gastrocnemius-plantaris, then for soleus. Length of record: 60 min. Between A and B: 40 min.

C. 40 min. later. Length of record: 15 min.

D. 44 min. later. Stimuli off at signal.

The slowest frequency at which the 4th and 5th stages became clearly recognizable was lower for soleus than for gastrocnemius-plantaris. Thus, at a frequency of 40 per sec. the typical sequence of phases of transmission was seen for soleus after about 6 hours of stimulation, whereas, during the

same period, the response of gastrocnemius-plantaris had only slowly declined to about 60 per cent of its initial magnitude.

With a frequency of 60 per sec., although the response of soleus was at first (3 to 5 min.) better sustained than that of gastrocnemius, total fatigue occurred sooner for the former than for the latter muscle. The 5th stage, on the other hand, began sooner for gastrocnemius-plantaris than for soleus. Figure 8 illustrates a typical observation.

At frequencies of 90 to 100 per sec. the differences in the behavior of the two muscles during the 4th stage were similar to those described for the frequency of 60 per sec. The 5th stage, however, began sooner for soleus than for the other muscle (table 2).

The tension developed during the 5th stage after equilibrium was attained was similar for the two muscles if referred to the maximal tension during the 1st stage as a basis for comparison (table 2).

I. *The results of brief (1 min. or less) rest-periods.* Such brief rest-periods were given in some of the first experiments made, in order to see whether the muscle would promptly relax to its resting tension, thus eliminating prolonged contraction-remainders or contractures. The effects recorded were later considered worthy of a systematic study.

It may first be stated that brief rest-periods do not influence significantly the time course of the 4th and 5th stages. In 3 animals records were taken from the same muscle on the two sides. One of the nerves was stimulated without interruptions, the other was allowed rest-periods of 30 or 60 sec. every 10 or 20 min. Save for the immediate effects of the removal of stimulation after the rest-periods the records were similar from the two sides.

At any time during the experiments even a very brief rest-period (1 sec. or less) resulted, upon renewal of stimulation, in an increment of the response, followed by a more or less rapid fall to the level preceding the interruption.

In the eight experiments in which the muscles were periodically allowed to rest for a given short time, the maximal tension attained upon renewal of stimulation varied characteristically as follows. The maxima decreased progressively throughout the 4th stage and also for some time after the beginning of the 5th stage (fig. 9). They later usually increased slightly as the tension corresponding to the 5th stage progressively increased. Thereafter the two tensions (maxima and basal tension of 5th) were approximately parallel. Figure 10 shows typical records. The lack of correlation between the recovery of the muscles during the rest-periods (as indicated by the maxima upon renewal of stimulation) and the progress of the 4th and 5th stages (as indicated by the tension existing when stimulation was interrupted) is further illustrated in figure 11. The break in the curve of the maxima during the 4th stage was present in 4 of the 8 experi-

ments made. In all 8 experiments the decline of the maxima persisted for some time after the beginning of the 5th stage.

J. *The subsidence of the 5th stage with rest.* In the previous section it was shown that if a brief rest-period is given after the neuro-muscular trans-

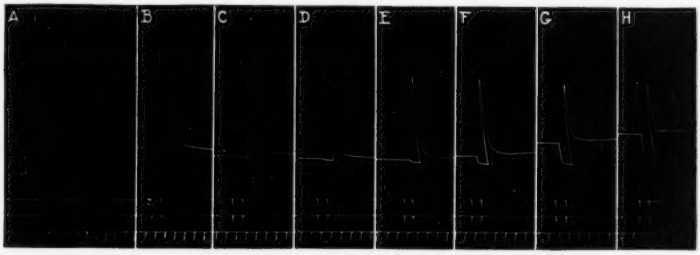

Fig. 9. Brief rest-periods during prolonged stimulation. Record from gastrocnemius-plantaris muscle. Frequency of maximal indirect stimulation: 60 per sec. The popliteal nerve was stimulated for periods of 14 min. separated by 1-min. resting intervals. Time signal: 1 min.

A. Beginning of stimulation.

B. to H. Rest-periods corresponding to successive 30-min. intervals after beginning of stimulation.

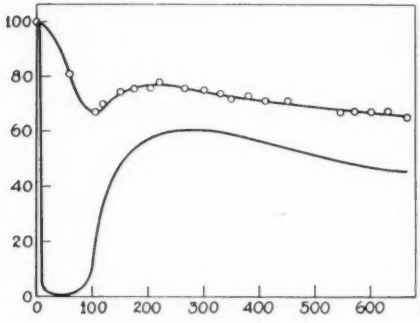

Fig. 10

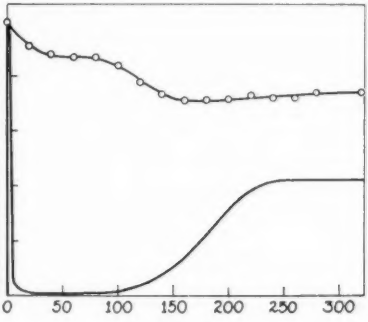

Fig. 11

Fig. 10. Mechanical responses of gastrocnemius-plantaris-soleus to prolonged, maximal indirect stimulation at 55 per sec. with interposed 1-min. rest-periods. Upper curve (circles): maximal tension attained upon reapplication of stimuli after a rest-period. Lower curve (continuous line): stable level of tension reached by the muscle after subsidence of the prompt initial rise resulting from the rest-periods (see fig. 9). Abscissae: time in minutes. Ordinates: tension expressed as per cent of the maximal tension attained at the beginning of the experiment.

Fig. 11. As in figure 10, but for a soleus muscle.

mission has reached the 5th stage, although there is a transient increase of tension above the previous level when the stimulation is renewed, the response rapidly recovers this previous level (fig. 9). This behavior may

be otherwise described by stating that the 5th stage does not disappear with brief (up to 1 min.) rest-periods. The present section deals with the influence of longer rest-periods upon the 5th stage. The purpose of these observations was mainly to investigate the stability of this stage and to find out whether or not in the process of its disappearance the neuromuscular system would pass again through the 4th stage or revert directly to the original fresh condition.

Two procedures were followed. In the first, records were taken from either gastrocnemius-plantaris or soleus on both sides. After several hours of stimulation with a given frequency and consequent full development of the 5th stage, different rest-periods were allowed on the two sides—e.g., 10 and 20 min., respectively. The second procedure consisted in giving increas-

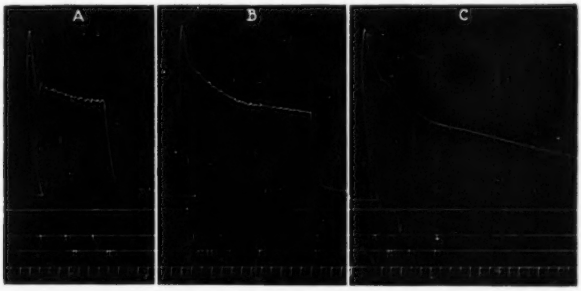

Fig. 12. Progressive subsidence of the 5th stage with rest. Record from gastrocnemius-plantaris. The nerve had been stimulated for 5 hours at 60 per sec.; the 5th stage had been fully attained. The response in A was obtained by reapplying the stimuli after 10-min. rest; the 5th stage was present undiminished. Between A and B, 15 min. additional rest were allowed; the 5th stage had only partially subsided. Between B and C, 25 min. more rest were given; the 5th stage disappeared almost entirely.

ingly longer rests to a muscle after it had reached the 5th stage, stimulating from 15 to 30 min. between these rest-periods.

The results may be summarized as follows. The 5th stage subsides progressively (fig. 12) and disappears within from 15 to 60 min. If after this disappearance stimulation is renewed, the entire sequence *1* and *3* → *4* → *5* will again occur but the 4th stage is usually briefer than originally; accordingly the 5th stage begins sooner (fig. 13).

Regardless of the length of the rest-period allowed, if the stimuli are reapplied the prompt fall to a very low tension characteristic of the 4th stage (fig. 9) is never observed. It may be therefore concluded that during the subsidence of the 5th stage the neuro-muscular junctions do not pass through the 4th stage, but enter the fresh condition directly.

The nerve and muscle action-potentials were recorded in several animals

during the reappearance of a 4th and 5th stage after recovery from a previous period of prolonged stimulation. The nerve spikes usually varied only slightly throughout this second period of stimulation (see section D). The muscle spikes showed again changes similar to those described in section E.

Discussion. I. *The 4th and 5th stages are synaptic phenomena.* The term "fatigue" is applied to a heterogeneous group of phenomena which have one feature in common: decreased activity in consequence of previous action. For accurate analysis it is necessary to determine where and how the impairment of reactivity has taken place. Even in a relatively simple

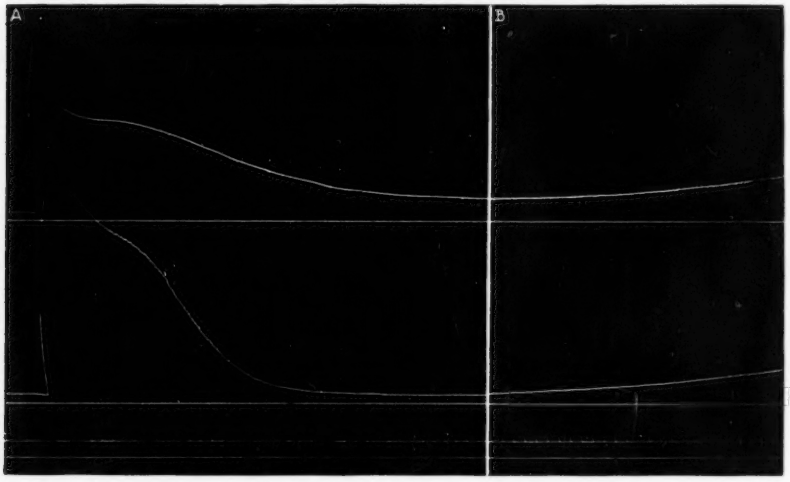

Fig. 13. Reappearance of the 4th and 5th stages after a prolonged rest. The records are from the experiment whose beginning is illustrated in figure 8. After the 4 hours of continuous stimulation shown in that figure a rest of 20 min. was given. The stimuli were then reapplied at A. Between A and B, 30 min. Time signal: 1-min. intervals.

system such as a nerve-muscle preparation, it appears as if at least two different types of fatigue may occur: a contraction- and a transmission-fatigue.

Since a denervated muscle stimulated directly may give evidence of fatigue, it is obvious that a depressed reactivity because of previous action exists, in which the nerves, and the nerve-muscle junctions play no rôle. It is probable, as had often been suggested, that such fatigue is due to the chemical changes attending muscular contraction. We propose to call this fatigue the *contraction-fatigue.*

The fatigue (4th phase) studied in the foregoing observations, on the other hand, is probably a junctional, synaptic phenomenon—i.e., a *trans-*

mission-fatigue. Kronecker and Gotch (1880) reported that the fatigue of indirectly tetanized muscles is independent of the load, and dependent only on the frequency of stimulation. The results described in sections F and G confirm this report. Indeed, with higher frequencies fatigue occurs sooner than with lower rates of stimulation and the muscle therefore develops less tension with the higher than with the lower frequencies. These facts exclude contraction and its chemical phenomena as the source of this type of fatigue. The experiments reported in a previous communication (Luco and Rosenblueth, 1939) are even more conclusive in showing that relatively rapid rates of stimulation result in a fatigue of the transmission process, not of the muscle. In those experiments prolonged stimulation of a motor nerve was started after a paralyzing dose of curare had been injected. Control stimulation of other nerves indicated when the curare had been entirely destroyed or eliminated. At the time of complete decurarization the continuously stimulated muscle showed deep neuromuscular fatigue. Such fatigue had taken place without any contraction of the muscle.

The evidence indicates that not only the 4th phase, but also the 5th stage is a synaptic phenomenon. The lack of participation of the nerve impulses will be discussed below. The purely muscular phenomena are eliminated by reasons similar to those mentioned above. The load—i.e., the amount of energy liberated by the muscle—has no influence on either the time course or the relative magnitude of the 5th phase (section F). Furthermore, with a high frequency of stimulation the appearance of the 5th stage is quicker than with a slower frequency (fig. 6), although the initial contraction is less for the high than for the slow frequency. Finally, since the 5th stage succeeds a prolonged period of lack of contraction, it seems evident that contraction should not be significant for its appearance.

II. *The 4th and 5th stages are independent.* By this statement is meant that the two stages are due to different and independent factors. The main argument in support of this conclusion is that whatever the factor or factors responsible for the impairment of transmission during fatigue, they are brought about by prolonged, rapid stimulation. Continued stimulation should, therefore, in all probability maintain or increase the process—i.e., a similar or deeper fatigue should ensue, not a renewed transmission—unless new factors come into play.

The paradoxical discrepancy between the two curves plotted in figures 10 and 11 is a further support for the conclusion under discussion. The maximal tension developed at the onset of further stimulation after a brief rest-period is a direct measure of the degree of recovery which takes place during the rest. It is therefore an indirect measure of the degree of fatigue—i.e., the greater the fatigue the less the recovery during a fixed period of rest. The more stable level of tension which the muscle promptly

attains when the stimuli are re-applied is, on the other hand, an indication and a measure of the prevalent 4th or 5th phase (fig. 9). The paradoxical discrepancy in question is the period during which the maxima continued to decrease while the steady tension was increasing with the beginning of the 5th phase. The previous interpretation leads to the inference that a decrease of the maxima denotes increasing fatigue. During this period, therefore, both fatigue and the 5th phase increase simultaneously. This inference is explicable if the conclusion of the independence between the two phases is accepted.

Further evidence for this independence is provided, finally, by the data on the disappearance of the 5th phase (section J). If the same factor or factors were responsible for the 4th and 5th stages the 4th phase should succeed the 5th during the subsidence of the latter, but such is not the case (fig. 12).

III. *The nerve action-potentials.* Although the records taken (figs. 1, 3 and 4) show the behavior of the A axons before their fine terminal branching it is likely that the nerve terminals differ only quantitatively from the axons from which they derive—i.e., the changes might be more marked at the nerve endings, but the axon spikes should indicate the general trend of these changes. On such a basis it may be concluded that neither the 4th nor the 5th stages are caused by variations in the nerve impulses.

The decline of the nerve spike-potentials, greater for higher than for lower frequencies (table 1, figs. 3 and 4) is probably indicative of nerve fatigue. By this is meant that prolonged stimulation of nerve leads to a decrease of the magnitude of the spike-potential per fiber. Alternation because of changes of electrical threshold, or because of increased refractory period, is excluded by the negative results of intensification of the stimuli or of slowing of the frequency of stimulation (p. 43). A local damage to some of the nerve fibers at the site of the stimulating electrodes was also excluded in some experiments, when stimuli were applied through another pair of electrodes closer to the recording pair after the responses from the more distant electrodes had undergone a significant decline. Such stimulation through other electrodes did not, as a rule, result in any increase of the recorded spike-potentials.

Knowledge of the behavior of fatigued nerves is slight. In the present study this problem was only incidental. The suggestion made above is, therefore, only tentative. If the decline of the spike-potentials is interpreted as fatigue, then it may be stated that rest-periods as long as 30 min. lead only to slight and inconsistent recovery (p. 44).

Whatever the explanation for the occasional decline of the nerve action-potentials, several facts show that such a decline is not responsible for the changes in the muscular response. Entirely similar mechanograms were obtained both when the nerve spike-potentials showed no decline and in the

instances when a decline took place. The changes in the magnitude of the spikes, when present, were unrelated to the changes of muscular tension (fig. 3). Brief rest-periods during the prolonged stimulation led to a marked immediate increase of the muscular response with no corresponding increase of the nerve responses. The prominent alternations which the muscle electrograms showed during the 5th phase (fig. 5) were not present in the nerve electrograms. Finally, periods of rest sufficient for the disappearance of the 5th stage did not significantly or consistently change the nerve spikes; upon renewal of stimulation, however, the muscle repeated the original sequence of the several phases.

IV. *The muscle action-potentials.* Two interesting features stand out in the data of section E: the lack of parallelism between some changes in the muscle electrograms and the mechanograms, and the alternation of the electric responses. The first feature is beyond the scope of this discussion.

Two questions arise concerning the alternations, the mechanism and the cause. The changes in the magnitude and latency of the peaks of the electrograms (fig. 5, p. 45) are readily explained as due to the irregular participation or lack of such participation of different groups of muscle fibers for different nerve volleys. This explanation appears more likely than the alternative, that the magnitude or latency of the spike-potential may vary per fiber. Indeed, some observations made on the facial muscles, in whose electrograms several elements or groups of elements may be readily distinguished (Rosenblueth and Morison, 1937*b*) clearly supported the explanation proposed; during the 4th and presumably the 5th stages, a given spike irregularly appeared or failed to appear, with fairly constant magnitude and latency, while the nerve was regularly and continuously stimulated.

Such alternation may be accounted for by assuming that for a given muscle fiber the stimulus delivered by the nerve, supramaximal during the 1st and 3rd stages, becomes of just threshold value at some time during the 4th and 5th phases. Very minor variations in either the magnitude of such stimulus or the threshold of the muscle could then determine whether or not the fiber would respond to a given nerve volley.

The alternation in the muscle electrograms leads to the inference that when fatigue begins, the fall of tension corresponds statistically to a condition in which more fibers are dropping out than those which are being liminally stimulated. Conversely, the rise of tension at the beginning of the 5th phase is due to a larger number of fibers coming into the liminal range than the number which is becoming inactive for successive nerve volleys. Only two relatively steady states appear in the records, the period of maximal fatigue and the plateau at the peak of the 5th stage. These would correspond to dynamic equilibria, an approximately equal number of elements coming in and going out per nerve volley.

attains when the stimuli are re-applied is, on the other hand, an indication and a measure of the prevalent 4th or 5th phase (fig. 9). The paradoxical discrepancy in question is the period during which the maxima continued to decrease while the steady tension was increasing with the beginning of the 5th phase. The previous interpretation leads to the inference that a decrease of the maxima denotes increasing fatigue. During this period, therefore, both fatigue and the 5th phase increase simultaneously. This inference is explicable if the conclusion of the independence between the two phases is accepted.

Further evidence for this independence is provided, finally, by the data on the disappearance of the 5th phase (section J). If the same factor or factors were responsible for the 4th and 5th stages the 4th phase should succeed the 5th during the subsidence of the latter, but such is not the case (fig. 12).

III. *The nerve action-potentials.* Although the records taken (figs. 1, 3 and 4) show the behavior of the A axons before their fine terminal branching it is likely that the nerve terminals differ only quantitatively from the axons from which they derive—i.e., the changes might be more marked at the nerve endings, but the axon spikes should indicate the general trend of these changes. On such a basis it may be concluded that neither the 4th nor the 5th stages are caused by variations in the nerve impulses.

The decline of the nerve spike-potentials, greater for higher than for lower frequencies (table 1, figs. 3 and 4) is probably indicative of nerve fatigue. By this is meant that prolonged stimulation of nerve leads to a decrease of the magnitude of the spike-potential per fiber. Alternation because of changes of electrical threshold, or because of increased refractory period, is excluded by the negative results of intensification of the stimuli or of slowing of the frequency of stimulation (p. 43). A local damage to some of the nerve fibers at the site of the stimulating electrodes was also excluded in some experiments, when stimuli were applied through another pair of electrodes closer to the recording pair after the responses from the more distant electrodes had undergone a significant decline. Such stimulation through other electrodes did not, as a rule, result in any increase of the recorded spike-potentials.

Knowledge of the behavior of fatigued nerves is slight. In the present study this problem was only incidental. The suggestion made above is, therefore, only tentative. If the decline of the spike-potentials is interpreted as fatigue, then it may be stated that rest-periods as long as 30 min. lead only to slight and inconsistent recovery (p. 44).

Whatever the explanation for the occasional decline of the nerve action-potentials, several facts show that such a decline is not responsible for the changes in the muscular response. Entirely similar mechanograms were obtained both when the nerve spike-potentials showed no decline and in the

instances when a decline took place. The changes in the magnitude of the spikes, when present, were unrelated to the changes of muscular tension (fig. 3). Brief rest-periods during the prolonged stimulation led to a marked immediate increase of the muscular response with no corresponding increase of the nerve responses. The prominent alternations which the muscle electrograms showed during the 5th phase (fig. 5) were not present in the nerve electrograms. Finally, periods of rest sufficient for the disappearance of the 5th stage did not significantly or consistently change the nerve spikes; upon renewal of stimulation, however, the muscle repeated the original sequence of the several phases.

IV. *The muscle action-potentials.* Two interesting features stand out in the data of section E: the lack of parallelism between some changes in the muscle electrograms and the mechanograms, and the alternation of the electric responses. The first feature is beyond the scope of this discussion.

Two questions arise concerning the alternations, the mechanism and the cause. The changes in the magnitude and latency of the peaks of the electrograms (fig. 5, p. 45) are readily explained as due to the irregular participation or lack of such participation of different groups of muscle fibers for different nerve volleys. This explanation appears more likely than the alternative, that the magnitude or latency of the spike-potential may vary per fiber. Indeed, some observations made on the facial muscles, in whose electrograms several elements or groups of elements may be readily distinguished (Rosenblueth and Morison, 1937*b*) clearly supported the explanation proposed; during the 4th and presumably the 5th stages, a given spike irregularly appeared or failed to appear, with fairly constant magnitude and latency, while the nerve was regularly and continuously stimulated.

Such alternation may be accounted for by assuming that for a given muscle fiber the stimulus delivered by the nerve, supramaximal during the 1st and 3rd stages, becomes of just threshold value at some time during the 4th and 5th phases. Very minor variations in either the magnitude of such stimulus or the threshold of the muscle could then determine whether or not the fiber would respond to a given nerve volley.

The alternation in the muscle electrograms leads to the inference that when fatigue begins, the fall of tension corresponds statistically to a condition in which more fibers are dropping out than those which are being liminally stimulated. Conversely, the rise of tension at the beginning of the 5th phase is due to a larger number of fibers coming into the liminal range than the number which is becoming inactive for successive nerve volleys. Only two relatively steady states appear in the records, the period of maximal fatigue and the plateau at the peak of the 5th stage. These would correspond to dynamic equilibria, an approximately equal number of elements coming in and going out per nerve volley.

V. *The differences between gastrocnemius-plantaris and soleus.* As is well known, the two muscles differ quantitatively in many respects. These differences are summarized by the statement that gastrocnemius-plantaris is a "fast" muscle, while soleus is a "slow" muscle. It is of interest to see whether or not the present data on the 4th and 5th stages can be correlated with the other characteristics of the muscles which have led to that systematization.

As reported in section G a certain relatively low frequency of stimulation which may be sufficient to bring about a marked transmission-fatigue in soleus may be incapable of producing similar fatigue of gastrocnemius after several hours of application. This fact would suggest a relative slowness of the neuro-muscular synapses of the slower muscle as compared to the faster gastrocnemius. With a higher frequency (e.g., 60 to 100 per sec.) maximal fatigue occurs earlier for soleus than for gastrocnemius—i.e., such frequencies are again relatively faster for the slow than for the fast muscle. It may be remarked that although gastrocnemius is still developing a significant tension when soleus has already attained a quite low level, the situation is reversed earlier during stimulation, the response of soleus being at first better sustained than that of gastrocnemius (fig. 8). This discrepancy may be due to a faster rate of contraction-fatigue and a slower one of transmission-fatigue, for gastrocnemius than for soleus.

The parallelism found above breaks down for other data. Thus, although the 5th stage develops sooner for gastrocnemius than for soleus at 60 per sec. (fig. 8), the reverse is found at higher frequencies (table 2). A more complete study, including other muscles, is necessary before any generalization on this aspect of the problem will be well founded. The purpose of recording separately from the two muscles in this study was to justify the conclusion that the 5th stage appears readily in both slow and fast muscles. Concerning this conclusion the data leave no doubt.

SUMMARY

The responses of gastrocnemius-plantaris and soleus to prolonged (several hours) stimulation of the popliteal nerve with frequencies from 25 to 300 per sec. were studied in cats. The period of neuro-muscular fatigue (4th stage) is followed by a later rise of tension which can attain high values and be sustained for many hours (5th stage; figs. 1 to 4, 6, and 8 to 11).

The 4th and 5th stages are uninfluenced by dial anesthesia (section B), by previous sympathectomy (section C) and by the load applied to the muscles (section F). They are influenced by the frequency of stimulation (section G; figs. 6 and 7). They are quantitatively different for gastrocnemius-plantaris and soleus (section H; fig. 8).

The changes of tension are not correlated with corresponding changes

in the motor nerve impulses (section D; figs. 1, 3 and 4). The changes in tension and in muscle action-potentials are roughly parallel (section E; figs. 2 and 4). The irregularities in the mechanograms during the 4th and 5th stages are due to alternation of the muscle fibers (fig. 5).

The effects of rest-periods of different durations are described (sections I and J; figs. 9 to 13). The 5th stage subsides progressively (figs. 12 and 13). The muscle does not revert to the 4th stage during this subsidence (p. 51).

It is inferred that the 4th and 5th stages are synaptic phenomena (p. 52); it is also shown that they are due to different and independent factors (p. 53). Two types of fatigue are recognized: a contraction-fatigue (p. 52) and a transmission-fatigue (4th stage, p. 53). A mechanism for the alternation of muscle fibers is suggested (p. 55). Some differences between gastrocnemius-plantaris and soleus are discussed.

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NEUROMUSCULAR "TRANSMISSION-FATIGUE" PRODUCED WITHOUT CONTRACTION DURING CURARIZATION

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In 1890 Bowditch, studying the indefatigability of nerves, reported the following observations. A paralyzing dose of curare was injected into cats or dogs. When paralysis had occurred a cut motor nerve was stimulated peripherally. The stimuli were induction shocks at tetanizing frequency and were continued without interruptions. Two to five hours later irregular contractions appeared in the corresponding muscles. Bowditch inferred that the appearance of the motor responses coincided with the wearing out of curarization.

An interesting corollary of this report was pointed out to us by Dr. W. B. Cannon. If Bowditch's observations and inferences were accepted it would follow that neuromuscular transmission is not "fatigued" during the period of action of curare, for the frequency of stimulation used by Bowditch leads to practically total lack of transmission within $\frac{1}{2}$ to 1 hour in uncurarized animals. It would further follow that curare blocks the process of transmission at some point of the neuromuscular complex previous to the step at which lack of transmission from fatigue takes place in the uncurarized systems.

In a previous communication from this Laboratory (Rosenblueth and Morison, 1937) it was concluded that the absence of transmission both in fatigue and in curarization may be explained by the single assumption that the quanta of acetylcholine released by the nerve impulses are below the threshold of the muscle. After curare the insufficiency of the acetylcholine released would be due to an increased threshold of the muscle, while in fatigue the quanta of the mediator would be decreased.

According to the foregoing hypothesis fatigue should occur not only if the muscle contracts in response to the nerve impulses but also when the contractions are prevented by curare. The interpretation which Bowditch adopted for his observations obviously contradicts this hypothesis. It was deemed important, therefore, to repeat Bowditch's experiments and to make adequate controls in order to test the accuracy of his interpretation,

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and thus also to test the validity of the more recent theories of neuromuscular transmission.

METHOD. Cats were used, either anesthetized with dial (Ciba, 0.75 cc. per kgm. intraperitoneally) or decerebrated under a brief ether induction. The muscles studied were soleus or gastrocnemius-plantaris. The tendons were freed on both sides and attached to myographs. The legs were fixed by drills inserted into the tibiae. The responses were recorded on a kymograph, upward excursions denoting contraction. The muscles pulled against one or several heavy rubber bands, the records showing, therefore, mainly changes of tension.

For stimulation a pair of shielded silver electrodes was applied to the popliteal nerve immediately below the severed sciatic. When the action potentials of the nerve were recorded a similar pair of electrodes, placed 5 to 6 cm. lower, was connected to a capacity-coupled amplifier, and this in turn to a cathode-ray oscillograph.

The stimuli were thyratron discharges of a condenser at a frequency of 60 per sec. The shocks were sent to the primary coil of a transformer and the nerves were activated by the diphasic output of the secondary coil. The duration of these diphasic waves was about 2 msec. The stimulator used had 2 output channels discharging synchronously but with independent intensity control. Each of these channels was connected to a transformer. Interrupters placed in the secondary circuits permitted the stimulation of either of the two nerves whenever desired. The polarity of the shocks was selected which yielded a greater response with submaximal stimulation. The intensity was then adjusted so that it was slightly supramaximal both for the muscles and for the A spikes of the nerves. The supramaximality was periodically tested. Only rarely was it necessary to intensify the stimuli in the course of an experiment—i.e., the threshold of the nerves did not vary significantly for several hours in these experimental conditions.

The curare used was the crude Brazilian product. It was injected intravenously in doses barely sufficient for complete paralysis of the muscles. Artificial respiration was administered through a tracheal cannula until complete decurarization.

In 3 cats the abdominal sympathetic chains (from L1 to S2) were removed aseptically under ether anesthesia 7 to 12 days before the acute experiment. In 2 of these animals the adrenal glands were tied off through a midline abdominal incision at the beginning of the experiment.

RESULTS. The cats were prepared as described above. After adjusting the polarity and the intensity of the stimuli, 2 or 3 control responses were recorded by stimulating both sides for periods of 5 sec. (fig. 1A). Curare was then injected. Control stimuli were applied at 5-min. intervals to ascertain when complete paralysis had occurred. The first dose of curare

injected was sometimes insufficient to bring about this complete paralysis. In such cases a small additional dose was administered (fig. 1B). When total curarization had been obtained (fig. 1C) stimulation of one of the nerves was begun (fig. 1D) and continued thereafter without interruption.

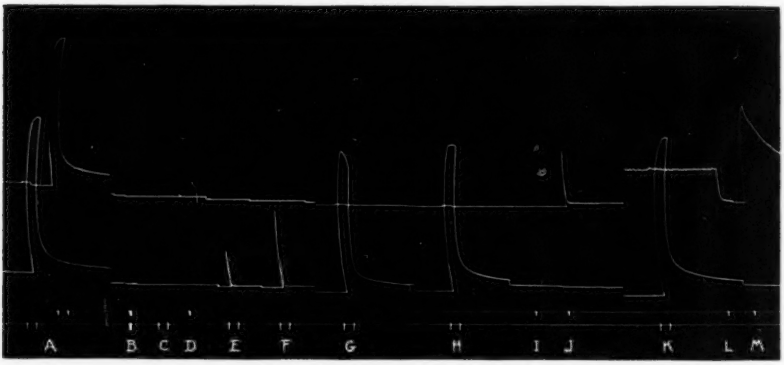

Fig. 1. Cat 8 in table 1. Abdominal sympathetic chains removed 12 days previously. Dial. Adrenals ligated. Gastrocnemius-plantaris (without soleus) recording on both sides. The upper signal marks the beginning or end of stimulation of the right popliteal nerve, which was activated continuously throughout curarization (upper record). The lower signal indicates the occasional tests made on the other control side (lower record).

A, 10:30 a.m. Five-sec. tests before curare. A first dose of curare was injected at 10:33; it did not produce complete paralysis of the muscles.

B, 10:56. Additional small dose of curare.

C, 10:59. Complete curarization.

D, 11:00. Beginning of prolonged stimulation of right nerve to muscle on the upper record.

E, 11:15; F, 11:30; G, 12:30 p.m. Increasing responses of control muscle showing progressive decurarization.

H, 12:59. Complete decurarization.

I, 1:00. Prolonged stimulation of right nerve stopped. The absence of a relaxation shows that no tension had developed throughout the period of stimulation.

J, 1:01. Stimulus reapplied to the right nerve. A comparison of this record with A shows the marked transmission-fatigue consequent to prolonged stimulation.

K, 3:56. The control response is equal to that at the end of decurarization (H). The continuously stimulated muscle is developing some tension (5th stage, see p. 61).

L, 3:57. Prolonged stimulation of right nerve stopped again.

M, 3:58. Stimulus reapplied to right nerve.

Since the animal was totally curarized such stimulation did not immediately result in any contraction.

The other muscle was used as an indicator of decurarization. Control 5-sec. periods of stimulation were applied to the nerve every 15 minutes.

Bowditch's statement that decurarization takes place very gradually was confirmed (fig. 1E, F, G). From 15 to 70 min. after the injection of curare (12 to 65 min. after complete curarization) the control muscle gave slight responses upon stimulation of its nerve, showing that transmission was already occurring for some of the fibers. Total recovery of the control responses was only evident, however, from 65 to 260 min. after the injections (fig. 1H).

The behavior of the muscle on the continuously stimulated side was quite different from that of the occasionally stimulated control side. In 7 out of the 10 animals studied the muscle on the continuously stimulated side was entirely quiescent at the time when the control responses showed complete decurarization—i.e., there was no measurable response to the nerve impulses (fig. 1I). In 2 other animals a similar complete failure of transmission was present when the control side showed 90 and 95 per cent decurarization, respectively. The last animal in the series died before complete decurarization (see below).

If at this stage of the experiments the continuous stimulation was stopped for 1 min. and then renewed, the corresponding muscle contracted. The response was, however, much smaller than that obtained in the tests before curarization and promptly subsided till complete disappearance within a few seconds (fig. 1J).

The persistent stimulation of one nerve was usually continued (or renewed after a 1-min. pause) for several hours after complete decurarization of the animal. It was then observed that the muscles gradually began to develop some tension (fig. 1K, L, M). This tension slowly increased until it reached a stable plateau which could be as high as 60 per cent of the test responses before curarization. Throughout this late development of tension irregularities (alternation) appeared both in the mechanogram (fig. 1K, L, M) and the muscle electrogram, much like the irregularities which Bowditch observed and attributed to the action of curare.

In a separate communication (Rosenblueth and Luco, 1939) we shall report a study of this late development of tension in muscles stimulated indirectly for long periods of time at fast frequencies. For present purposes it is sufficient to state that entirely similar delayed, irregular responses appear in animals which have not been curarized at all. We propose to speak of these late responses as the 5th stage of neuromuscular transmission. It is this 5th stage which Bowditch saw in his experiments and which he wrongly attributed to curare. It is also this 5th stage which had begun before complete decurarization in the 2 animals mentioned above (cats 2 and 4, table 1), in which contraction of the continuously stimulated muscle appeared when the control responses were 90 and 95 per cent, respectively, as high as the tests before curare. It may be repeated that since the 5th stage occurs in non-curarized animals it is irrele-

vant in a study of the influence of curare upon neuromuscular transmission.

The behavior of the continuously stimulated nerve was followed in 5 animals by recording periodically the A spike-potentials. In 2 of the 5 cats the magnitude of the spikes did not vary throughout the period of curarization. In the other 3 the nerve spike-potentials were 5, 10 and 20 per cent, respectively, lower at the time of total decurarization than they had been before the injection of the drug. This decline of the electric response was not due to submaximality of the stimuli or alternation of the nerve fibers, for neither intensification nor slowing of the frequency of stimulation resulted in any increase of the spikes.

Maximal stimulation of the popliteal nerve activates not only the motor fibers but also the vasomotor nerves distributing to the muscles. To test the possible influence of vasoconstriction on the results, the abdominal sympathetic chains were removed in 3 animals, as described under "method," and time was allowed for degeneration of the postganglionic fibers in the sciatic. In 2 of these cats the adrenal glands were ligated at the beginning of the experiment. In one of these animals the control muscle had recovered up to 80 per cent from curarization without any contraction of the continuously stimulated side. The untimely removal of the artificial respiration led to asphyxia and death. For this reason, this animal is not included in table 1. In the 2 other sympathectomized cats (fig. 1, cats 8 and 9, table 1) the results were entirely similar to those obtained in the unoperated animals.

The possibility that dial anesthesia might have an influence on the course of the experiments was eliminated by the typical results obtained in 3 decerebrate animals (cats 4, 5 and 6, table 1), the observations being started 1 hour after cessation of the brief ether induction under which decerebration was performed.

Table 1 summarizes the experiments carried out. It is clear that stimulation of motor nerves during the action of curare, although it does not elicit any contractions of the corresponding muscles, leads to transmission-fatigue at the end of curarization. The muscles develop little or no tension during uninterrupted stimulation after the effects of the drug have entirely disappeared in the control muscles. Even after a rest period of 1 min. the nerve-muscle subjected to prolonged stimulation reveals marked deficiencies of transmission when the delivery of the shocks is renewed. The failure of transmission is not due to a failure of the nerve fibers; it is not due to vasomotor phenomena caused by the activation of sympathetic fibers or by secretion of adrenaline; it is finally uninfluenced by dial anesthesia.

Discussion. These results do not contradict the observations of Bowditch. The controls made lead, however, to an interpretation different from that which Bowditch adopted. In the present experiments the doses of curare administered were usually adjusted to be barely sufficient

for complete paralysis of the muscles. Bowditch, on the other hand, being interested in the fatigability of nerve, not that of muscle, gave large doses of the drug in order to insure prolonged curarization. The late development of tension which is brought about by frequent repetitive stimulation of the nerve (5th stage, p. 61) is uninfluenced by previous administrations of curare. The appearance of this 5th stage was approximately coincident with the elimination or destruction of large doses of curare in Bowditch's experiments, but occurred as a rule some time after cessation of the effects of small doses of the drug in the foregoing observations (table 1). For this reason Bowditch failed to realize that neuromuscular fatigue may be produced without contraction during curarization.

TABLE 1

In the column headed "Muscle" the letters have the following meaning: G., gastrocnemius; P., plantaris; S., soleus. The end of decurarization was measured when the control responses were equal to the original tests before curare. The percentages refer to the original test responses as 100 per cent. The animals 4, 5 and 6 were decerebrated; the others were under dial anesthesia. In the animals 8 and 9 the abdominal sympathetic chains had been removed several days previously.

ANIMAL NUMBER	MUSCLE	BEGINNING OF DECURARIZATION	END OF DECURARIZATION	PER CENT RESPONSE OF CONTINUOUSLY STIMULATED SIDE AT END OF DECURARIZATION	PER CENT RECOVERY OF CONTROL AT BEGINNING OF 5TH PHASE	BEGINNING OF 5TH PHASE
		minutes	*minutes*			*minutes*
1	G.P.S.	30	90	0	100	140
2	G.P.S.	30	180	14	95	165
3	G.P.S.	70	180	0	100	200
4	G.P.	70	260	3	90	250
5	S.	70	120	0	100	140
6	S.	25	100	0	100	160
7	G.P.	35	65	0	100	125
8	G.P.	20	120	0	100	210
9	S.	15	135	0	100	165

The share of nerve in neuromuscular transmission-fatigue was early eliminated by the studies of Bernstein (1877), Wedensky (1884) and Bowditch (1890). Waller's (1885) observation that when a muscle no longer responded to motor nerve impulses it still would contract when stimulated directly, led him to suggest that failure of transmission of the nerve impulses was due to fatigue of the motor end plate. Lapicque and Lapicque (1919) interpreted neuromuscular fatigue as resulting from a decreased electrical excitability of the muscle fibers. Fulton (1926) adopted the same view and suggested further that this change of electrical excitability is produced by the accumulation of acid metabolites in the muscle during contraction. It is interesting to note that in making these suggestions

the Lapicques and Fulton overlooked the important observations of Kronecker and Gotch (1880), Wedensky (1891) and Hofmann (1904). Kronecker and Gotch showed that the fatigue of a tetanized muscle is only a function of the frequency of stimulation, not of the load; thus, transmission-fatigue should not be attributed to the muscular activity. Wedensky and Hofmann found no changes in the electrical excitability of fatigued muscles stimulated directly; they therefore suggested that the failure of transmission was due to fatigue of the nerve endings—an explanation similar to that proposed by Waller.

The present data are conclusive for the elimination of both the motor nerve impulses and the muscle as sources of the failure of transmission after prolonged repetitive stimulation. The nerve is excluded not only in the cases in which the spike potentials maintained their full magnitude but also when a small decline of the spikes was recorded (p. 62). In the latter instances a period of rest of 1 min. resulted in no change of the decreased nerve spike-potentials, yet the muscle gave an initial response upon removal of stimulation (fig. 1J). Thus, unless it should be assumed that the nerve impulse may vary in magnitude at the nerve ending independently of its magnitude in the rest of the axon—an unlikely assumption which would demand experimental proof—the failure of transmission at the end of curarization should not be attributed to a depression of the nerve impulses.

The muscle is even more directly excluded by the data as a factor in the appearance of transmission-fatigue. The possible influence of curare on muscular excitability would have entirely disappeared in figure 1H, since the control muscle showed a 100 per cent recovery of its response. If muscular activity were to play a rôle in transmission-fatigue the control muscle should be more depressed than the muscle on the continuously stimulated side, for the latter had not contracted at all between figure 1B and 1J, while the control muscle responded repeatedly during the tests for decurarization made systematically every 15 minutes.

It may be stressed that the foregoing results do not disprove the possibility that muscular contraction may have an influence upon the process of transmission. But they prove that it is possible to fatigue transmission without any contraction. Any theory of fatigue which does not account for that fact is therefore inacceptable.

The previous discussion leads to the conclusion that a failure of transmission of the motor nerve impulses may take place when neither the spike-potentials of nerve nor the electrical excitability of muscle are significantly modified. Such a conclusion is obviously incompatible with the electrical theory of transmission, according to which the nerve spike potential is the stimulus whereby the motor nerve activates the muscle fibers. The chemical theory of transmission, on the other hand, readily accounts for the data. If it is assumed that repetitive stimulation results in a reduction

of the quanta of acetylcholine liberated at the nerve terminals per nerve impulse, as stated in the introduction, the failure of transmission at the end of curarization can be attributed to a fall of the quanta of acetylcholine below the threshold of the muscle fibers.

This interpretation is fundamentally in agreement with Hofmann's (1902) theory of transmission-fatigue. The knowledge of chemical mediation of nerve impulses was then not available, yet Hofmann had the insight to realize that such fatigue (as opposed to contraction-fatigue), since it could not be attributed to the nerve impulses or the muscle, should be located at the nerve-endings.

SUMMARY

A motor nerve was continuously stimulated in cats with a frequency of 60 per sec. after a paralyzing dose of curare had been injected. When the control muscle of the opposite side, periodically tested, showed complete decurarization, the muscle on the continuously stimulated side was usually not contracting (fig. 1, table 1). A renewal of the prolonged stimulation after a rest of 1 min. revealed marked transmission-fatigue (fig. 1). The same results were obtained in animals anesthetized with dial and in decerebrate preparations (table 1). Previous (over 8 days) removal of the abdominal sympathetic chains and ligation of the adrenals (fig. 1) did not influence the phenomenon.

The similar experiments made by Bowditch (1890) are discussed and explained (p. 62). It is concluded that transmission of the motor nerve impulses may be fatigued in conditions in which neither the nerve spike-potentials nor the electrical excitability of muscle are impaired. This conclusion is incompatible with the Lapicques' (1919) and Fulton's (1926) theories of transmission-fatigue (p. 64). It is also incompatible with the electrical theory of neuromuscular transmission, but is readily accounted for by the chemical theory of mediation by acetylcholine (p. 64).

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DIFFUSION OF CALCIUM, MAGNESIUM AND PHOSPHORUS INTO THE PERITONEUM

The Effect of Intravenously Injected Calcium Salts and of Parathyroid Hormone

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Methods generally used for the estimation of the diffusible fraction of serum calcium are open to certain objections. In vitro studies involve the use of an artificial membrane of fixed permeability, whereas the permeability of the capillary walls varies in different situations and under different conditions. Moreover, the process of ultrafiltration usually takes 3 to 4 hours, during which time some readjustment of equilibrium between the two fractions in the serum may occur. The use of the cerebrospinal fluid calcium concentration as a measure of the diffusible fraction of serum calcium has been objected to on the ground that this fluid is probably not a simple dialysate or ultrafiltrate of the blood plasma. Edema fluids are not open to this objection. However, the mere presence of these fluids implies the existence of abnormalities of the forces which operate to maintain normal equilibrium at the capillary membrane. Such conditions obviously do not furnish a satisfactory basis for the study of the diffusibility of calcium under various superimposed experimental conditions.

In the present investigations an attempt is made to overcome these objections by studying changes in a fluid introduced into the peritoneal cavity of normal dogs. Changes in the concentration of calcium and other electrolytes in this fluid, after they have attained equilibrium with the blood plasma, may be representative of changes in their concentration in the interstitial fluid and may afford a more satisfactory index of the influence of various agents in this connection than that afforded by methods previously employed.

Methods. Normal, unanesthetized dogs (18–29 kgm.) were used in all cases. The fluid introduced into the peritoneal cavity was 2.5 per cent dextrose in 0.9 per cent NaCl solution. Blood samples were obtained from the femoral artery and samples of fluid were withdrawn simultaneously from the peritoneum after manipulation of the abdomen to facili-

tate uniform composition of the fluid. The animals showed no evidence of undue discomfort and were allowed to move about the laboratory during the experimental period.

Calcium determinations were made by the Clark-Collip (1) modification of the Kramer-Tisdall procedure following precipitation of the proteins by trichloracetic acid. Inorganic phosphorus was determined by the method of Fiske and Subbarow (2) and magnesium by the method of Haury (3). In the cases in which protein estimations were made, the method of Howe (4) was employed. Ultrafiltration was accomplished by the method of Greenberg and Gunther (5) with certain modifications suggested by Smith and Sternberger (6). The diffusible calcium of the serum was calculated from the calcium concentration of the ultrafiltrate by making a correction of 5 per cent for protein volume.

EXPERIMENTAL PROCEDURE AND RESULTS. *Experiment 1.* Three animals (dogs 1–3) received, intraperitoneally, 50 cc. and three animals (dogs 4–6) 100 cc. of fluid per kilogram of body weight. Blood was taken before and samples of blood and peritoneal fluid at ½, 1, 2, 3, 4, 5 and 24 hours after introduction of the fluid. Three animals (dogs 7–9) received 100 cc. of fluid per kilogram. After 17 hours, samples of fluid were withdrawn hourly for 7 hours.

The rates of diffusion of calcium and phosphorus into the peritoneal fluid are indicated by the data in table 1. It was found that if less than 50 cc. of the solution per kilogram was introduced it was impossible to obtain satisfactory samples after 18 hours. Comparison of the present data with those presented by Schechter, Cary, Carpentieri and Darrow (7) suggests that variation in the volume of peritoneal fluid between 40 and 100 cc. per kilogram has little significant influence upon the rate of establishment of equilibrium between this fluid and the blood plasma. It would appear, however, that the use of 50 cc. per kilogram is preferable for the study of early changes in the diffusion of calcium and phosphorus, particularly the latter, from the blood plasma. The data obtained in dogs 7–9 indicate that equilibrium between the blood and the peritoneal fluid, once established, remains constant under normal conditions during the experimental period (24 hrs.). In these animals, hourly examination of the concentration of calcium in the fluid between the 17th and 24th hours revealed a maximum variation of only 0.3 mgm. per cent. The protein content of the fluid did not change significantly during this period; in no instance did it exceed 0.75 gram per 100 cc. Variations in this factor may therefore be disregarded as contributing to variations in the concentration of calcium in the fluid.

Experiment 2. Three animals (dogs 10–12) received 100 cc. of fluid per kilogram intraperitoneally. Eighteen hours later samples of blood and fluid were withdrawn for analysis. Ten cubic centimeters of 20 per cent

calcium gluconogalactogluconate[1] were injected intravenously, followed immediately by intravenous injection of 400 cc. of a 2 per cent solution of this salt in Ringer's solution by continuous drip over a period of 2 hours in two cases and 1 hour in one case. In the latter instance, blood and

TABLE 1

Rate of diffusion of Ca and P after intraperitoneal injection of 2.5 per cent dextrose in 0.9 per cent NaCl solution

Dogs 1–3 received 50 cc. and dogs 4–6 100 cc. per kilogram of body weight.

DOG		HOURS							
		0	½	1	2	3	4	5	24
1	Serum Ca*	11.6		11.8		11.2		11.5	11.1
	Fluid Ca*		2.1	3.2	5.4	6.2	6.4	7.1	7.3
	Serum P*	4.2		4.1		4.4		4.6	4.3
	Fluid P*		1.2	2.8	3.4	4.0	4.2	4.4	4.4
2	Serum Ca	11.3		11.3		11.5		11.2	11.4
	Fluid Ca		1.7	2.8	4.6	5.7	6.3	7.0	7.0
	Serum P	4.1		4.3		4.3		4.5	4.7
	Fluid P		1.0	2.3	2.9	3.6	4.0	4.2	4.5
3	Serum Ca	11.0		11.2		11.6		11.0	11.3
	Fluid Ca		1.9	3.3	4.5	5.8	6.0	6.8	7.2
	Serum P	4.2		4.0		4.2		4.4	4.5
	Fluid P		1.3	2.6	3.1	3.8	3.9	4.0	4.2
4	Serum Ca	11.6		11.2		11.0		11.4	11.0
	Fluid Ca		2.0	3.1	3.6	4.2	5.8	6.7	6.9
	Serum P	5.1		4.9		5.2		5.5	5.6
	Fluid P		1.1	1.9	2.1	3.0	4.2	5.0	5.2
5	Serum Ca	10.3		10.5		10.2		10.6	10.8
	Fluid Ca		1.6	2.6	3.2	3.8	5.7	6.3	6.6
	Serum P	4.0		4.2		4.2		4.5	4.7
	Fluid P		0.9	1.6	1.8	2.1	3.2	4.0	4.5
6	Serum Ca	10.7		10.2		10.5		10.7	10.3
	Fluid Ca		1.2	2.3	3.1	3.9	5.7	6.2	6.8
	Serum P	4.2		4.3		4.7		4.4	4.6
	Fluid P		1.1	1.6	1.9	2.3	3.5	4.1	4.5

* All values expressed in milligrams per 100 cc.

fluid samples were withdrawn after 15, 30, 45 and 60 minutes; in the two former, samples were withdrawn after 30, 60, 90 and 120 minutes. In

[1] The calcium gluconogalactogluconate was obtained from the Sandoz Chemical Works through the courtesy of Mr. Harry Althouse.

one case (dog 12) the diffusible fraction of the serum calcium was determined by ultrafiltration. In this case, additional samples of blood and fluid were withdrawn at 180 minutes, one hour after cessation of calcium injection.

The findings were of essentially the same order in the three animals. The serum calcium rose from an average of 10.4 mgm. per cent to 14.6 mgm. at 30 minutes and then gradually to 16.3 mgm. at 120 minutes. Little significant change occurred in the peritoneal fluid calcium during the first 30 minutes; it then rose gradually, the average increase being 1.9 mgm. at 120 minutes. Consequently, the fluid: serum calcium ratio fell sharply during the first 30 minutes and then increased gradually. The average values were as follows: control, 67 per cent; 15 minutes, 47 per cent; 30 minutes, 49 per cent; 60 minutes, 55 per cent; 120 minutes, 55 per cent. In one animal (dog 12) studied 60 minutes after cessation of calcium administration, the serum calcium had fallen to 13 mgm. (from 16.1 mgm. at 120 min.) and the fluid : serum calcium ratio had risen to 64 per cent despite the fact that the concentration of calcium in the peritoneal fluid had fallen simultaneously (from 9.2 mgm. at 120 min. to 8.2 mgm.).

Smith and Sternberger (6) found that when the serum calcium was increased by the intravenous injection of diffusible calcium salts the proportion of diffusible calcium (ultrafiltration) at the peak of the serum calcium was lower than previously in 17 of 18 cases. As the serum calcium concentration fell the proportion of diffusible calcium increased, usually reaching the control level in about 5 hours. The data obtained in dog 12 are in accord with these findings. The diminution in the proportion of diffusible calcium was somewhat more prolonged, a circumstance which may be due to the maintenance of hypercalcemia by continuous injection. Comparison of the calcium content of the peritoneal fluid with that of the artificial ultrafiltrate in dog 12 reveals that the former lags behind the latter during the periods of both increasing and decreasing values. The difference is slight, however, and is in accord with what might be expected on the basis of previous observations regarding the delayed establishment of calcium equilibrium between the plasma and the peritoneal fluid.

The question naturally arises as to whether the calcium content of the peritoneal fluid is representative of that of the interstitial fluid of the body under conditions of constant and of changing serum calcium concentration. Two logical objections may be raised to this assumption: *a*, the peritoneal space is not in direct contact with the capillary endothelium (peritoneum and omentum); *b*, diffusion may occur more rapidly into normal tissue spaces, with their small fluid content, than into the large volume of fluid introduced into the peritoneal space. However, the fact seems significant that certain constituents of the plasma, such as the

nonprotein nitrogen, come into equilibrium with the peritoneal fluid with great rapidity (15–30 min.) (7) whereas the establishment of calcium equilibrium requires 3 to 6 hours. This suggests that the factor of fluid volume may not be of as much importance as might be supposed and that diffusible constituents of the plasma may pass into the interstitial fluid at different rates.

Experiment 3. Ten adult dogs (16–28 kgm.) (dogs 13–22) were given intraperitoneal injections of the dextrose-saline solution (100 cc. per kgm.). After 16 hours, blood and fluid samples were withdrawn. Five hundred or 600 (3 cases) units of parathyroid hormone[2] were then injected intramuscularly. Blood and fluid samples were obtained simultaneously hourly for 8 hours for calcium, phosphorus and magnesium estimation. In 5 cases additional samples were withdrawn after 24 hours. In 2 instances estimations were made also of the diffusible fraction of serum calcium by ultrafiltration through a collodion membrane (5).

Relatively slight changes occurred in the serum magnesium concentration. This is in accord with previous observations (8, 9). There was, however, a rather constant moderate increase in the fluid magnesium concentration, resulting in an increase in the fluid:serum magnesium ratio from an average control level of 78 per cent to a maximum of 83 to 102 per cent at 6 hours, followed by a slight decrease. The serum and fluid phosphorus concentrations fell practically simultaneously in 5 animals, suggesting a tendency toward a continuous diffusion equilibrium between the fluid and the blood plasma. The drop in the former lagged behind that in the latter, resulting in a distinct increase in the fluid:serum phosphorus ratio during the first 4 hours after parathyroid administration (average control ratio, 90 per cent; average at 4 hours, 100 per cent).

The maximum increase in serum calcium occurred consistently much earlier (average increase 2.1 mgm. at 5–6 hrs.) after parathyroid administration than is usually the case (10–18 hrs.). The reason for this cannot be stated, unless it be the large amount of hormone, given intramuscularly. Previous studies (10, 11, 12, 13) indicated that the administration of this agent results in a practically proportionate increase in diffusible and nondiffusible serum calcium, as determined by artificial membrane methods. In the two instances in which this method was employed in the present study (fig. 1), the diffusible fraction increased disproportionately during the period of increasing calcemia, the percentage diffusibility continuing to increase during the 2 hours of diminishing serum and ultrafiltrate calcium concentrations. The diffusibility ratio was essentially normal at the end of 24 hours. The changes in serum and peritoneal fluid calcium in the 8 animals in this group are exemplified by the data in

[2] The parathyroid hormone was supplied by Dr. E. A. Sharp, of Parke, Davis and Company.

2 animals presented in figure 1. These findings indicate that under conditions of changing serum calcium concentration the ultrafiltrate calcium is not accurately representative of the calcium content of the artificial peritoneal fluid. This discrepancy is due apparently to the fact that the equilibrium between the fluid and the plasma, particularly in the case of calcium, is not established promptly in the face of changes in either medium. Consequently, the rise and fall of calcium concentration in the fluid lags somewhat behind that of diffusible calcium in the blood.

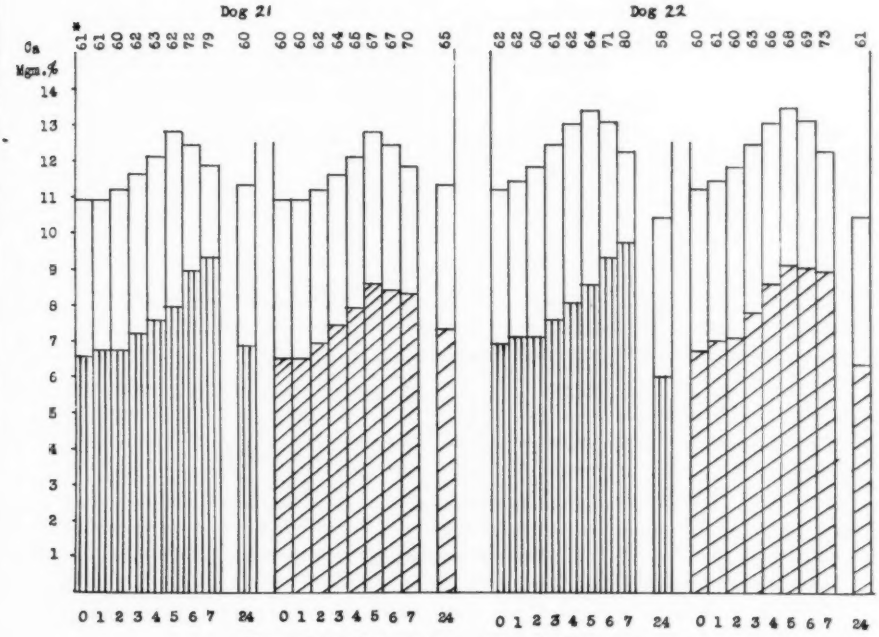

Fig. 1. Vertical lines represent peritoneal fluid calcium. Diagonal lines represent ultrafiltrate calcium (collodion membrane).

* Ratio (per cent) diffused to total serum calcium.

It would seem preferable to distinguish, therefore, between "diffusible" and "diffused" calcium, magnesium and phosphorus, changes in the former being an index of the direction but not necessarily of the extent of changes in the latter during periods of changing serum values.

Experiment 4. Two adult dogs (18 kgm.) (dogs 23, 24) were subjected to repeated (7 times each) filling and emptying (after 17 hrs.) of the peritoneal cavity (dextrose-saline solution) during the preceding 3 weeks. They were then treated as indicated in figure 2. The purpose of the

repeated filling and evacuation of the peritoneal cavity was to determine whether the removal, by this means, of an unusual amount of diffusible calcium from the body would influence the response to parathyroid hormone.

These data support the general belief that the primary effect of the parathyroid hormone upon serum calcium is to increase its diffusible component, the rapidly established equilibrium between the latter and the nondiffusible fraction resulting in an almost immediate proportional increase in both fractions. The validity of this hypothesis has not been

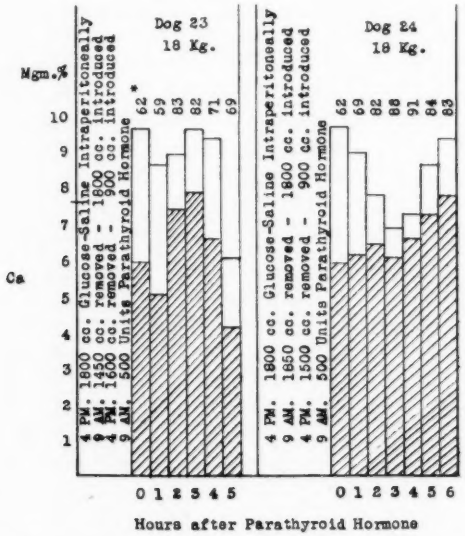

Fig. 2. Changes in serum and peritoneal fluid calcium following the injection of 500 units parathyroid hormone in animals in which the peritoneum had been repeatedly filled and evacuated prior to the administration of the hormone.

* Ratio of peritoneal to serum calcium expressed as percentage.

definitely established. One of the chief sources of difficulty lies in the fact that when hypercalcemia is produced by the intravenous injection of a completely diffusible calcium salt the proportion of diffusible serum calcium is not only not increased but is usually temporarily diminished. The observation that the concentration of calcium in the peritoneal fluid actually increased considerably after injection of parathyroid hormone in the presence of a constant (dog 23) and a falling (dog 24) serum calcium concentration (fig. 2) suggests that the fundamental effect of the hormone on the serum calcium is to increase its diffusion from the blood into the

interstitial fluid. Under normal conditions this is believed to be secondary to an increase in the diffusible fraction of serum calcium, resulting from active mobilization of calcium from the bones, with consequent hypercalcemia. Under such circumstances, the maintenance of the normal ratio of diffusible to total serum calcium is due perhaps to prompt "binding" of some of the mobilized calcium by serum protein or in a "colloidal calcium phosphate complex," masking the primary nature of the increase in the diffusible fraction. The data presented in figure 2 suggest the possibility that the parathyroid hormone may increase the diffusible calcium at the expense of the nondiffusible fraction under certain conditions. Why the production of this phenomenon should have been effected by the removal from the body of such relatively small amounts of calcium as 183 mgm. (dog 23) and 201 mgm. (dog 24) under the conditions of the experiment cannot be stated at the present time. It is of interest also that no manifestations of tetany developed in these animals despite the rather marked fall in serum calcium (to 6.05 and 6.89 mgm. per 100 cc.).

SUMMARY AND CONCLUSIONS

1. Calcium and phosphorus equilibrium between the blood plasma and fluid introduced into the peritoneal cavity is reached in 4 to 5 hours and the calcium equilibrium remains constant during the remainder of the 24 hour period.

2. When hypercalcemia is produced and is maintained by the continuous intravenous injection of a diffusible calcium salt, the proportion of diffusible serum calcium, as determined by ultrafiltration, is diminished, tending to return to normal as the serum calcium concentration falls. The calcium content of the ultrafiltrate increases steadily during the period of maintained hypercalcemia.

3. The increase in the diffusible fraction of serum calcium is followed, after about 30 to 45 minutes, by a progressive increase in the concentration of calcium in the peritoneal fluid, the values for the former and the latter being essentially identical at 1 to 2 hours. During the period of diminishing calcemia the value for peritoneal fluid calcium falls more slowly than that for the ultrafiltrate calcium.

4. Following the administration of parathyroid hormone, the ratio of peritoneal fluid calcium to serum calcium remains approximately constant for about 5 hours. Subsequently, as the serum calcium falls, this ratio increases, reaching a maximum at the end of the experimental period (7–8 hrs.). The rise and fall in peritoneal fluid calcium lags behind that of the diffusible fraction of serum calcium as determined by ultrafiltration.

5. Under certain conditions the administration of parathyroid hormone may result in hypocalcemia. A significant increase in the calcium con-

tent of the peritoneal fluid ("diffused" calcium) may occur under such circumstances in the presence of a constant or a falling serum calcium concentration. These findings suggest that the fundamental effect of the hormone on the serum calcium is to increase its diffusion from the blood into the interstitial fluid.

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THE EFFECT OF CARMINE UPON THE GASTROINTESTINAL MOTILITY OF CHILDREN

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One requisite of the metabolic balance method of studying the utilization of foods by man or animals is separation of the feces which correspond to the food ingested during a given interval of time. To accomplish this it is customary to have the subject ingest an inert, colored substance such as wood charcoal or carmine at the beginning and end of the period to be studied. The "marker" changes the color, and in some cases the consistency, of a section of the feces and renders a separation and differentiation possible. In addition, the time elapsing while a marker traverses the digestive tract may be used as a measure of gastrointestinal motility.[1]

The fact that in some cases carmine *does* cause a marked change in the consistency of the feces, suggests that it may also increase gastrointestinal motility to the point of interfering with maximum digestion and utilization. The present roentgenologic study has been designed to determine whether the carmine used to demark the feces, and thereby ascertain metabolic balances at 5-day intervals, influenced the rate of gastrointestinal motility of seven children who had been subjects of a metabolic study of growth extending over eight successive months.[1] By observing the serial order of events portrayed in roentgenograms taken at frequent intervals following the consumption, alone, of a barium-milk meal and, at a different time, the same meal plus carmine, one is able to compare the emptying times of the stomach, the different segments of the intestinal tract, the types of dispersion of the meals, the lengths of exposure of the meals to the various digestive and absorptive processes, and the speeds of their passage throughout the alimentary tract.

The subjects of the study were known to be representative of average well children, ages 7 to 11 years, having in their medical history no record of abnormal hereditary influence or excessive illnesses. They approximated the standards (1, 3) of weight for height (table 1).

Previous to the study, the children had been subjects of extensive metabolic observations and all were standardized in their living habits

[1] A monograph on the chemistry of growth is in preparation.

and accustomed to experimental routine, in which they coöperated with a genuine interest in its successful culmination. The children lived together as one family with a housemother in a separate, modern home in the country and had ample opportunity for recreation, indoors and out, and lived in a comfortable, serene and homelike atmosphere. Their dietary, physical routine, and environment were comparable with those of the average school child in an adequate "home" situation.

PROCEDURE. Two test meals were given three weeks apart, each consisting of 2 ounces of barium sulfate in 4 ounces of pasteurized milk. The meals were given at body temperature and immediately preceding the second one, 0.2 to 0.3 gram of carmine was ingested. The experimental (carmine) meal has been contrasted with the control meal of barium and milk alone. The subjects were not permitted to ingest any food after the evening meal upon the day before a roentgenographic series was to be initiated. Laxatives were not given before or during the studies, nor were

TABLE 1

CHILD	AGE	SEX	RECUMBENT LENGTH	WEIGHT	PHYSICAL TYPE $\sqrt[3]{\frac{\text{WEIGHT}}{\text{RECUMBENT LENGTH}}}$
	mo.		*cm.*	*kgm.*	
D. P.	135	M	152.0	39.92	0.0226
W. P.	131	M	132.0	35.27	0.0248
H. H.	120	M	139.0	32.43	0.0229
F. C.	114	M	134.0	30.39	0.0234
R. S.	102	M	132.6	26.42	0.0224
B. M.	99	F	123.2	23.81	0.0234
J. H.	82	M	125.5	25.86	0.0236

any other preliminary or preparatory procedures followed. The activities, environment and diets (comparable to the diets ingested during the eight continuous months of the metabolic study (2)) of the children were interfered with only upon the first morning of each series. The children were taken from their cottage to the roentgen-ray laboratory, without breakfast or ingestion of any liquids upon the first morning of the study. The first roentgenogram of each child was taken within ten minutes after ingestion of the barium meal and films exposed at approximately half-hour or hour intervals until the entire meal had emptied from the stomach. During this interval the children were closely supervised; they were allowed to play in the laboratory but were restrained from eating or drinking until a roentgenogram showing the stomach empty was procured. Roentgen observations were made 24 and 48 hours, approximately, after ingestion of the meal and, in the instances in which the entire meal had not reached the rectum, an exposure was made at the 72-hour interval.

The roentgen examinations were made with the subjects in prone ventral position with the target 31 inches from the film and centered on the first to second lumbar vertebra; exposure was $\frac{1}{5}$ sec. at kv. peak of 92 and 50 ma. with Potter-Buckey diaphragms. Patterson par-speed screens were used for the smaller children and speed screens with the larger ones. The children had been subjects of previous roentgenographic studies and their gastrointestinal patterns had been determined with a barium and water meal and found to be within normal limits.[1] They were thoroughly conversant with the technique from frequent practice trials and were not, therefore, subject to any obvious psychological embarrassment which might alter the gastrointestinal response and thereby vary the pattern.

During the 26-day period within which four roentgenographic serial studies were made at weekly intervals (the two reported herein being the first and last) the time of occurrence of each defecation was recorded for all of the children. The regular elimination habits of the children are shown by the average of 1.4 bowel movements per child per day which is in

TABLE 2

BARIUM-MILK MEAL GIVEN	GASTRIC EMPTYING TIME (MINUTES)							
	D. P.	W. P.	H. H.	F. C.	R. S.	B. M.	J. H.	Average
Alone (control)	230	270	265	240	290	270	240	257
Preceded by carmine	130	190	130	190	220	190	190	177
Per cent decrease in emptying time	43	30	51	21	24	30	21	31

excellent agreement with the general average of 1.8 defecations for the same children during the previous metabolic study over eight continuous months.

Results. The gastric emptying times of the children, estimated from the series of roentgenograms, was less in all of the subjects when the test meal was preceded by carmine (table 2). The average decrease was 31 per cent; the range 21 to 51 per cent.

The increased emptying times of the stomachs are further shown in figure 1. The first exposures for all the children showed that in the same length of time (approximately 10 min.) the carmine meal had progressed much further than the control. Apparent in each of the carmine series was an immediate ejaculation of contents from the stomach and a rapid diffusion throughout the entire small intestine, in definite contrast to the control roentgenographs.

The progress of the control and carmine meals, at intervals, is shown in figure 1, and the tendency of the carmine meal to move much more rapidly for the first one and one-half hours after ingestion is quite clear.

After 90 minutes it is evident that the differences in progress were lessened but the carmine meal was definitely further along in the tract than the control meal. After three hours both meals were massed in the lower ileal loops or in the cecum and ascending colon. Although, for the group, the roentgenograms after this interval still showed the carmine meal to be slightly more advanced than the control, it would seem that the carmine

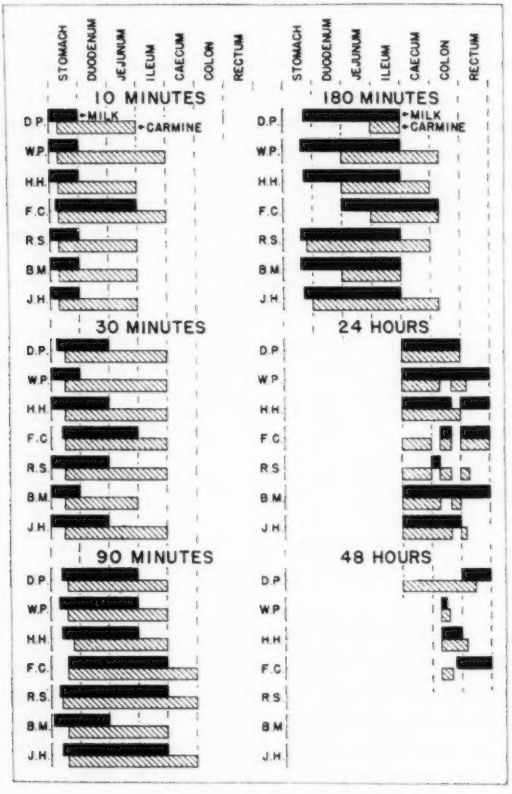

Fig. 1

meal was slowed or the control meal speeded up in this section of the tract. This is further evidenced by the fact that after 48 hours the control meal showed greater progress for three of the four children who had some of the meal left in the tract.

Upon the 24 hour exposures the individual variations in response were more apparent, although, in general, the rates of motility shown after this

interval compared rather closely with the per cent decrease in emptying time of the stomach and seem unrelated to the length of time which the stomachs actually required to empty. At the end of 48 hours the same general picture remained for all of the children. The tracts of F. C., J. H. and R. S. were practically empty; those of B. M. and W. P. showed faint traces; and D. P. and H. H. showed a much larger amount remaining in the colon and rectum. At the end of 72 hours all of the tracts were empty except that of D. P. and H. H. with the control meals, in which some material could be seen in the rectum.

Each child had a bowel movement upon the morning preceding both study periods; six of the seven children had bowel movements the first evening of the control series; the other child had had a movement the evening previous and had one early the next morning. One child had a bowel movement in the morning before the meal was given following

TABLE 3

Total defecations of seven children for first 4 days of observation periods

AFTER INGESTION OF	DAY MEAL WAS GIVEN	DAY AFTER MEAL WAS GIVEN	THIRD DAY	FOURTH DAY	TOTAL	AVERAGE PER CHILD PER DAY
Milk + barium	6	10	9	11	36	1.28
Milk + barium + carmine	7	9	12	6	34	1.21

TABLE 4

Average number of defecations per day for first 4 days of control and carmine observation periods

PERIOD	D. P.	W. P.	H. H.	F. C.	R. S.	B. M.	J. H.
Milk + barium	1.0	1.25	1.0	1.25	1.5	1.25	1.75
Milk + barium + carmine	1.0	1.5	1.0	1.0	1.75	1.25	1.0

carmine; the other six had movements that evening. The bowel movements recorded for the days the meals were ingested and the three following days are shown in table 3.

During the entire 26 days of supervision, within which the two study periods occurred, the average number of defecations per child per day was 1.4. The fact that the averages for the first four study days of each period (table 4) are less than this figure may indicate either the effect of disruption of the children's routine or a constipating effect of the barium meal; however, the small numbers of movements upon the first days of the studies are no doubt attributable, in part at least, to the psychological effect of disrupting the routine of the children to bring them to the laboratory for study, and to the experimental routine upon the first examination day which excluded all foods from the children, including liquids, until the middle or late afternoon. The intake for that day was considerably de-

preciated with a resultant reduction in bulk throughout the tract. A slight laxative effect might be surmised from the increased number of bowel movements upon the third day after ingestion of the carmine followed by a 50 per cent decrease in the number the next day. During the control period the reverse occurred, an increase in number of defecations from the third to the fourth day.

The seven subjects each excreted the marker in the second defecation following its ingestion. The elapsed time for six of the children was within a range of ten minutes, for the other child the interval was, roughly, a full 24 hours longer.

As may be noted there was an increase in the number of defecations coincident with the taking of carmine for two children, a decrease for two, and no change for three, when compared with the barium-milk meal alone. When the numbers of bowel movements were averaged for all subjects for each of the two series, i.e., with the barium-milk meal and the barium-milk-carmine meal, there were slightly fewer for the latter, 1.28 and 1.21 respectively. There was an average elimination rate of 1.4 defecations per child per day during the 26 days within which four barium milk meals were given, only one of these preceded by carmine. The barium apparently slowed the gastrointestinal motility of the subjects for, under the same environmental conditions, during a continuous metabolic study over eight continuous months, during which the subjects received no barium or cathartics, the average number of defecations per child per day was 1.8. This is in accord with the common observations on the constipating effect of barium sulfate.

The carmine tends to speed up gastric emptying time and delay the movement of the meal through the intestines, factors which somewhat equalize each other in the rate of passage of the meal through the entire tract thus yielding a normal gastrointestinal motility as measured by the carmine marker method. The fact that carmine does cause some deviation from the usual gastrointestinal pattern of children, emphasizes the need of extending the metabolic balance over a sufficient number of days to permit all of the carmine to be passed out and its effect removed, before terminating the period. Balances of sufficient length for gastrointestinal adjustment are essential in accurate metabolic determination.

From observations herein recorded there is a shortened peptic and a lengthened tryptic digestion. Although the results of this study do not permit a statement on the effect of shortened peptic digestion and lengthened tryptic digestion upon metabolism, the subject is worthy of further investigation. In the same vein we might ask also whether the carmine in its stimulation of a rapid gastric emptying time has altered the absorptive processes in the remainder of the alimentary tract due to the shortened period the food has been exposed to peptic digestion; and in the cases

where there is a like rapid emptying of the jejunum might not this alter the absorptive process and effect the metabolic balance?

SUMMARY

Roentgenographic study of the effect of 0.2 to 0.3 gram of carmine upon the gastrointestinal motility of seven average, healthy children, ages seven to eleven years, indicated that the carmine had little effect upon the total time of retention of a test meal (two ounces barium; four ounces water) in the tract. However, definite changes in the motility of the sections of the tract were produced by the carmine. The average emptying time of the stomach was 257 minutes with the test meal alone; with the test meal preceded by the carmine the average was 177 minutes. The per cent decrease in emptying time of the stomach with the carmine meal ranged from 21 to 51 per cent, average 31 per cent.

The roentgenograms indicated that, after leaving the stomach, the test meal without carmine tended to increase the speed of its passage until, about four or five hours after ingestion of the meal, the progress made by the two meals was approximately equal. Exposures 48 hours after ingestion of the meals showed little, if any, effect of carmine upon the emptying times of the entire tract.

Complete records of the time of each defecation during the 26 days within which four test meals were followed roentgenographically, did not show variations which could be attributed to the carmine. The average of 1.4 bowel movements per day per child compared favorably with the average of 1.8 for the same children during a previous metabolic study over eight consecutive months during which a carmine marker of the same quantity was given each fifth morning to separate the fecal units of each balance period.

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THE EFFECT OF BILE ON THE PROPULSIVE MOTILITY OF THIRY-VELLA LOOPS IN DOGS

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The cathartic action of bile is commonly attributed to a stimulating influence of bile salts on the large intestine, an influence which is illustrated by the fact that defecation follows their introduction into the colon or rectum (7, 17). Similarly the presence of constipation which accompanies a biliary fistula has been attributed to the lack of a normal stimulating action of bile on the colon (13). Thus Sobotka (19) suggests that in line with the classification of Meyer and Gottlieb (11) bile salts should be listed with those cathartics which exert their effects through stimulation of the large intestine. As a result of the observations of Galapeaux, Templeton and Borkon (6), however, there is some doubt that bile exerts its effect through a stimulation of the colon. By means of the balloon method these workers found that although defecation occurs within a few minutes following the introduction of bile into the dog's colon, the predominant response is a depression of motility which lasts 50 to 100 minutes.

The effect of bile or bile salts on the motility of the small intestine has been studied most commonly by means of excised intestinal strips suspended in suitable saline baths. It has been claimed repeatedly that the motility of such strips is inhibited following the application of bile (3, 4, 18). Several of the experiments of Ott and Scott (14) in which a similar method was used, suggest that bile inhibits strips of both small and large intestine. The significance of these findings is made questionable in the light of the experiments of Nakata (12) in which the application of bile to the serous surface of a strip inhibited activity while its application to the mucous surface gave little effect. The work of Schüpbach (17) on two Vella loop dogs suggests that bile inhibits peristaltic motility of the small intestine, but the extremely slow rate of propulsion observed both before and after the application of bile would seem to indicate that his loop preparations were not sufficiently motile for such a study. These

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and other similar findings have led to the belief that bile exerts either no effect or an inhibitory influence on the motility of the small intestine (19).

On the other hand, Horrall and his associates (8) have furnished evidence that bile stimulates the small intestine. As the result of a series of experiments which involved the use of multiple tandem balloons and various types of intestinal fistulas in dogs, these workers conclude that the small intestine is stimulated not only by artifically administered bile but also by bile which flows via the normal route into the duodenum. In agreement with this result is that of Fubini and Luzzati in 1888 (5) in which the propulsion of a pea through an intestinal loop was found to be accelerated following the introduction of bile.

The advantages of the bolus method of studying the propulsive motility of the small intestine have been pointed out by Quigley, Highstone and Ivy (15). Since previous reports based on this method are not in agreement and since any action of bile or its constituents on the propulsive motility of the small intestine may well be of fundamental importance in the normal physiology of the gastrointestinal tract, it has seemed desirable to subject the problem to further study.

METHOD. A series of Thiry-Vella loops was prepared in healthy adult dogs. The openings in the abdominal wall were made large enough to insure the ready entry and exit of a pellet after the loop ends had healed in place. All loops were so arranged that the distance between the exposed ends was approximately 12 cm. Care was taken at the time of operation to pull the loop ends out far enough to avoid the possibility of kinks. End-to-end anastomosis was made according to the method described by Martzloff and Burget (10). Since loops which were permitted to go unused for several days in succession often exhibited a marked diminution in motility, it was necessary to begin using them daily within 8 to 10 days after operation.

The rate of propulsion was determined by means of a sponge rubber pellet measuring approximately 0.8 cm. by 2 cm. The time elapsing between the introduction of the pellet at the proximal end of the loop and its appearance at the distal end was taken as propulsion time. In the process of introducing the pellet great care was taken to avoid undue mechanical stimulation.

Each dog was trained to lie quietly and to submit without excitement to all of the procedures incident to an experiment. After a preliminary control period during which normal propulsion time was determined repeatedly until 3 fairly consistent results were obtained, 2 cc. of the solution to be tested were pipetted at body temperature into the proximal end of the loop. Immediately thereafter the pellet was reinserted and the propulsion time again determined three times in succession.

RESULTS. I. The effect of various saline solutions used as controls.

a. In order to determine whether the manipulation incident to the introduction of a given solution into a loop might be responsible for changes in the rate of propulsion, a series of determinations was made before and after the introduction of 2 cc. 0.9 per cent NaCl. In a group of 27 experiments on 5 dogs only an occasional slight change was noted following the introduction of the saline. A typical experiment yielded normal propulsion rates of 4, 4, and 3.5 min. as compared with 3.7, 5, and 5.5 min. after the saline. Considered statistically the data indicate that the average difference in propulsion time before and after the introduction of the saline is only 0.3 times its sigma. Therefore any slight effect of the solution in a given experiment is due merely to the operation of chance.

b. The possibility that limited variations in ionic concentration of solutions might account for changes in propulsion time was investigated by the use of a series of NaCl solutions of 0.45, 0.9 and 1.8 per cent respectively. All were buffered with phosphate to a pH of 6.4, a figure which is compatible with those cited in the literature for dog's gall bladder bile (19) and with those of our own determinations. A series of 6 experiments on 2 dogs for each of the above salt solutions failed to demonstrate any influence which could be attributed to variation in ionic concentration. A typical experiment for any one of the three solutions resulted in normal propulsion times of $1\frac{1}{2}$ min., 2 min. and 2 min. as compared with rates of 2, $1\frac{1}{2}$, and 2 min. after the introduction of the salt solution.

c. Since the pH of dog's gall bladder bile has been reported to vary from a low of 5.2 to a high of 6.97 (19), it is desirable to study the effect of limited variations in pH on the propulsive motility. Accordingly observations were made on the effect of four 0.9 per cent NaCl solutions which were buffered with phosphate to a pH of 4.8, 5.6, 6.4 and 7.6 respectively. The results of 6 tests on 2 dogs for each of the solutions indicate that pH variations within the range used are without significant influence on the propulsive motility. An experiment which is typical for any of the solutions used resulted in normal propulsion times of $2\frac{1}{2}$, 2, and 2 min. as compared with rates of 2, $2\frac{1}{4}$, and 2 min. after the introduction of the buffered saline solution.

II. *The effect of bile.* The introduction of dog's gall bladder bile into the proximal end of the loops was followed by a marked increase in propulsive motility in all but 2 of 27 experiments on 5 dogs. A typical experiment furnished normal readings of 4, 6, and 5 min. as compared with readings of 2, 3, and 3 min. after the introduction of bile. A statistical analysis of the data reveals that the average lowering of the propulsion time is 5 times its sigma. Therefore the effect of bile on the rate of propulsion is a real one. Samples of bile obtained from the gall bladders of several dogs were found to have no apparent difference in their ability to

increase the rate of propulsion. In 12 of the experiments the bile was diluted to twice its volume with 0.9 per cent NaCl without noticeably altering its effectiveness. Table 1 presents the results of 5 representative tests using dog's gall bladder bile.

III. *The effect of bile salts.* In a series of 21 experiments on 4 dogs using 82 parts of bile salts[2] per 1000 parts of distilled water as well as in 8 experiments on 5 dogs using a solution of 164 parts per 1000 the propulsion time was without exception markedly reduced. The concentrations of these solutions are within the range reported for dog's gall bladder bile (19). Since bile salts are only weakly dissociated, the latter solution should be nearly isotonic with physiological saline. Table 1 includes the

TABLE 1

Propulsion rates in minutes before and after the introduction of the solutions indicated. Five typical results are given for each solution

DOG NO.	NORMAL RATES OF PROPULSION			PROPULSION RATES AFTER 0.9% NaCl			DOG NO.	NORMAL RATES OF PROPULSION			PROPULSION RATES AFTER DOG'S GALL BLADDER BILE		
1	2.0	1.5	1.5	2.5	2.5	2.0	1	5.0	4.5	5.5	2.0	2.7	3.2
3	8.0	8.5	10.0	11.0	11.0	13.0	3	7.0	7.0	8.0	2.5	3.0	3.5
4	2.6	2.8	2.5	3.2	2.8	2.5	5	2.2	3.2	3.0	1.7	1.7	1.8
5	4.0	4.0	3.5	3.7	5.0	5.5	6	3.0	5.0	5.0	2.0	2.0	1.5
6	3.6	2.5	2.8	2.6	2.2	2.8	2	4.0	3.5	3.5	2.0	1.5	2.0
				PROPULSION RATES AFTER BILE SALTS*							PROPULSION RATES AFTER BILE PIGMENT		
1	4.5	4.0	5.0	0.5	1.0	0.5	1†	5.5	8.0	5.0	5.0	5.0	4.0
2	3.0	4.0	4.0	1.5	1.7	0.7	2‡	1.0	1.0	1.0	1.7	1.2	1.5
3	8.0	8.0	9.0	1.5	2.0	1.5	3‡	5.0	4.5	5.0	5.0	4.0	6.5
4	1.5	1.2	1.2	0.3	0.5	0.5	5†	3.5	3.0	4.0	3.5	3.2	3.0
5	2.7	2.7	3.0	1.8	1.2	2.5	3‡	3.0	2.0	3.5	3.5	2.5	3.5

* Mixture of taurocholate and glycocholate.

† Bilirubin solution was used.

‡ Biliverdin solution was used.

data obtained from 5 representative experiments on bile salts. In an additional series of 12 experiments on 2 dogs using solutions of purified sodium taurocholate[3] in concentrations of both 41 and 82 parts per 1000, and of 9 experiments on 1 dog using purified sodium glycocholate[3] in a concentration of 82 parts per 1000, propulsion time was again markedly reduced in every case. An experiment which is typical for all of the bile salt solutions resulted in normal propulsion times of 5, 5, and 5 min. as

[2] A product of Merck and Company containing a mixture of taurocholate and glycocholate.

[3] Products of Pfanstiehl Chemical Company.

compared with times of 1.5, 1, and 1 min. after bile salts. Considered statistically the average change is 4 times its sigma.

IV. *The effect of bile pigments.* A 0.5 per cent solution of each of the bile pigments was prepared by dissolving in weak alkali and buffering with phosphate to the lowest pH compatible with solubility of the pigment (2). In 10 experiments on 3 dogs using bilirubin and 11 experiments on 4 dogs using biliverdin, the average change in propulsion time when considered statistically was only 1 times its sigma. An experiment which is typical of either group of experiments on these pigments yielded propulsion times of 5, 4½, and 5 min. before, and 5, 4, and 6½ min. after the introduction of bile pigment. The phosphate solution used in buffering the bile pigment solutions was likewise found to be without effect in 22 experiments on 4 dogs, the average change in propulsion rate being only 0.2 times its sigma.

V. *The effect of a calcium chloride solution.* A 0.05 per cent solution of calcium chloride in physiological saline furnishes a concentration of calcium ions which is within the range reported for gall bladder bile (19). In 28 experiments on 5 dogs, the average change in propulsion time was less than 1 times its sigma. A typical experiment resulted in propulsion times of 3, 4, and 3½ min. before, and 4, 4, and 3 min. after the introduction of the calcium solution.

VI. *The effect of mucin.* A solution of 16 parts of mucin per 1000 parts of water was found to have no significant effect on the propulsive motility in 22 experiments on 4 dogs. A typical experiment resulted in propulsion times of 3, 4, and 3 min. before, and 3, 4, and 3½ min. after the introduction of the mucin. Considered statistically the average change in propulsion time was less than 1 times its sigma which indicates that the solution has no real effect.

VII. *The effect of unsplit fats.* The exclusion of bile from the small intestine is known to result in the occurrence of increased amounts of unsplit fats in that organ. In order to determine whether the presence of such fats might be responsible for changes in the rate of propulsive activity of the intestine, a series of 23 determinations on 5 dogs was made before and after the introduction of corn oil. A typical experiment yielded readings of 5, 6, and 5 min. before, and 8, 7, and 8 min. after the introduction of the oil. Statistically the average change in rate was found to be only 1 times its sigma, which indicates that the propulsive motility of Thiry-Vella loops is not influenced by the presence per se of unsplit fats.

Discussion. The data presented indicate that the introduction of bile into the proximal end of a Thiry-Vella loop is promptly followed by an increase in propulsive motility. Of the several constituents of bile

which were studied only bile salts were found to have a similar effect. Thus the stimulating action of bile is readily accounted for on the basis of its bile salt content. This would seem to indicate that cholesterol as well as several other constituents of bile which were not included in this study do not contribute significantly to the influence of bile on propulsion. The data also indicate that a classification of cathartics which includes bile salts with those which exert their influence on the large intestine only is not justified.

In view of the gastric inhibition which is promoted by virtue of the presence of fats in the duodenum (16) it would seem to be desirable from the standpoint of gastric emptying that fat be rather promptly removed to more distal portions of the small intestine. The presence of fat in the duodenum is also associated with an increased flow of bile from the gall bladder (9). It may well be that the resultant increase in its bile content results in a physiological stimulus to the duodenum and that this action serves to facilitate the movement of fatty chyme into the jejunum, and indirectly to aid in removing the gastric inhibition which is promoted by the presence of such chyme in the duodenum. It is suggested that the delay in gastric emptying which occurs as a result of biliary obstruction may be due in part to the failure of the normal stimulating action of bile salts on the propulsive motility of the upper small intestine.

As the chyme progresses down the intestinal canal bile salts are gradually absorbed. It seems probable that this loss of bile salts may be associated with the progressive decline in the rate of propulsion which is said to occur (1). Thus bile salts may account in part at least for the fact that chyme travels more rapidly in proximal than in distal portions of the small intestine.

SUMMARY

The influence of bile and several of its constituents on the propulsive motility of the dog's small intestine has been studied by determining the time taken for the passage of a sponge rubber pellet through Thiry-Vella loops. The introduction of dog's gall bladder bile into the proximal end of a loop is promptly followed by a marked increase in the rate of propulsion. A solution of bile salts is fully as effective as bile. Several other normal constituents of bile as well as appropriate control solutions failed to influence propulsive motility. It is concluded that the application of bile to the mucous surface of the dog's small intestine results in an increase of propulsive motility and that the stimulating influence of bile salts accounts for this effect. It is suggested that bile salts may play an important rôle in the normal regulation of the propulsive movements of the small intestine.

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THE ECONOMY OF EFFORT INDEX FOR HEARTS OF NORMAL AND HYPERTENSIVE SUBJECTS

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An estimate, even if a somewhat approximate one, as to how the efficiency of the left ventricle in patients with hypertension compares with that of normal individuals is not only of practical, but also of physiological importance. Nevertheless, even an approach to such an evaluation has always proved difficult. By using a heart-lung preparation or similar artificial circuits which permit calculations of the total energy liberated by the heart muscle and the work realized, Evans (1), Starling and Visscher (2), Peters and Visscher (3), Katz and Mendlowitz (4), among others, have been able to study the efficiency of the mammalian heart under a variety of conditions. The technical difficulties of obtaining similar data in the intact animal and the virtual impossibility of studying cardiac efficiency in this manner in man are obvious.

A possible approach to the problem was suggested by Wiggers and Katz (5) in 1928. They postulated that *during the phase of ejection* the left ventricle expends energy *a*, as static effort in maintaining pressure at a diastolic level, and *b*, as dynamic effort in ejecting blood under pressure into the aorta. To determine the relative values for the two energies they divided an intraventricular pressure curve as shown in figure 1 and measured areas B and D with a planimeter. The quotient $\frac{\text{area D}}{\text{area B}}$ was regarded as an index of the economy of effort during ejection. While such a quotient is not equivalent to that expressing the mechanical efficiency of the heart beat $\left(\frac{\text{work energy}}{\text{total energy}}\right)$ studies (5) of the energetics of muscular contraction suggest that the two may alter in the same direction, though not quantitatively, under varying dynamic conditions.

The present communication deals with an application of the procedure to man for the purpose of studying the variations that exist in this "economy of effort" quotient in normal individuals and in patients with hypertension.

METHOD. Our method is based on the assumption that the contour

of a human subclavian pulse curve from the inception of the rise to the beginning of the incisura represents the ejection phase of the human intraventricular pressure curve and that if ordinate pressure values can be supplied the ejection phase of the intraventricular pressure curve can be reconstructed with reasonable accuracy both as to contour and pressure values. This assumption rests predominantly on the premise defended by Wiggers (6) and recently supported by Gregg, Eckstein and Fineberg (7) that the left intraventricular pressure curve is virtually the same as the aortic in contour and only slightly greater in magnitude at homonymous points. That the degree of difference may vary somewhat in the subjects studied and that this constitutes an imperfection in our

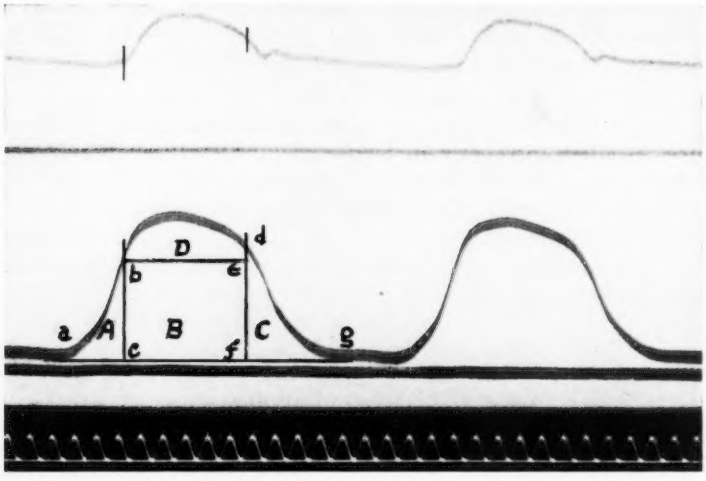

Fig. 1

procedure is recognized but we believe the error incurred is not sufficient to invalidate the broad conclusions reached.

The following procedure was adopted. Subclavian pulse curves were recorded using a Frank segment capsule and only such curves were utilized as showed essentially the same ordinate level at the onset and termination of each complete cycle. Immediately after obtaining the pulse record the brachial blood pressure was determined by the Korotkoff method using the fourth phase for the diastolic pressure. As shown in the original curves (fig. 2, lower curves—reduced in size for reproduction) these values can be introduced at the lowest and highest points on the pulse curve signifying lowest diastolic and highest systolic pressures respectively. Because of the variation in sensitivity of the capsules application of the

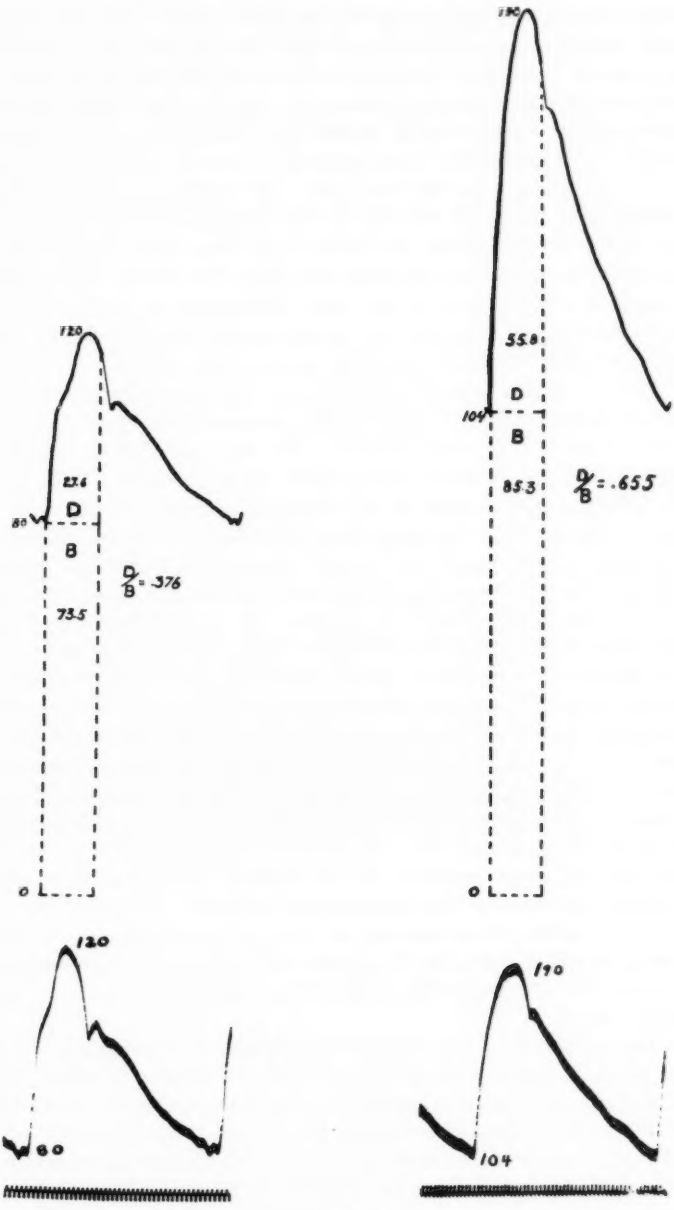
120
80
27.6
D
B
73.5
D/B = .376
0
190
104
55.8
D
B
85.3
D/B = .655
0
120
80
190
104

Fig. 2

receivers, etc., the amplitude of the original curves represents varying ordinate values. For purposes of visual comparison the originals in figure 2 (lower half) were enlarged to two and one-half times their size and corrected to the same ordinate scale (fig. 2, upper half) using the coördirectograph described by H. Green (8). Using the scale of pressures established by applying the blood pressure values to the subclavian pulse curve it is possible to plot the zero level. By dropping perpendiculars at the beginning and end of ejection to the zero line and drawing a line parallel to the zero line from the onset of ejection, these curves, like the intraventricular curves are divisible into areas B and D. The ratio of the measured areas B and D was then determined as in figure 2. All of the subclavian pulse records were obtained with the subject in a sitting position and after a twenty to thirty minute rest period.

Material. Eighty-one individuals comprising chiefly medical students, instructors and attendants in good health constituted our group of normal subjects. Nine of these were females. The ages varied from twenty-one to sixty-nine, fifty individuals being below 30 years of age. The hypertensive group was composed of fifty-four individuals selected from the dispensary and wards of Lakeside Hospital with no particular attempt to classify them as "essential" or "renal" types. All had blood pressure readings of at least 160 mm. Hg systolic and 100 mm. Hg diastolic pressure for a known period of at least six months. Of these thirty were females and the group spread from 22 to 69 years in age, twelve being under forty. Two of this group exhibited moderate congestive heart failure and a few complained of substernal distress or paroxysmal dyspnea. Of the patients studied seven have since died (indicated by small triangle in fig. 3).

Results. The data obtained by analyzing the curves from these subjects as indicated were methodically studied in regard to various conditions and it was found that the significant results could be plotted on one chart, shown in figure 3. A solid vertical line indicates the systolic, diastolic (3) and pulse pressures of the normal subjects and the dotted lines, similar pressures for the hypertensive subjects. For each line a dot represents the index for the economy of effort of the normal subject and a small cross the same index for the hypertensive subjects. The cases were arranged so that in proceeding from left to right they have indices of ascending value.

Discussion of data for normal subjects. It is apparent from a consideration of figure 3 that the calculated economy of effort varies widely in the case of normal subjects. The extremes of the index range from 0.215 to 0.880 and the median was 0.428. Careful study of the data showed that the index is greater in individuals having a large pulse pressure, a relatively low diastolic pressure or a combination of the two. This is in accord with experimental studies of Wiggers and Katz (5)

who found that the chief factors influencing the index are the diastolic pressure and the degree of initial stretch of the ventricular wall which manifested itself in greater systolic discharge and larger pulse pressure. It may well be therefore that normal subjects with essentially the same diastolic pressures may have sufficient differences in initial ventricular stretch—either as a result of different rates of venous return or different

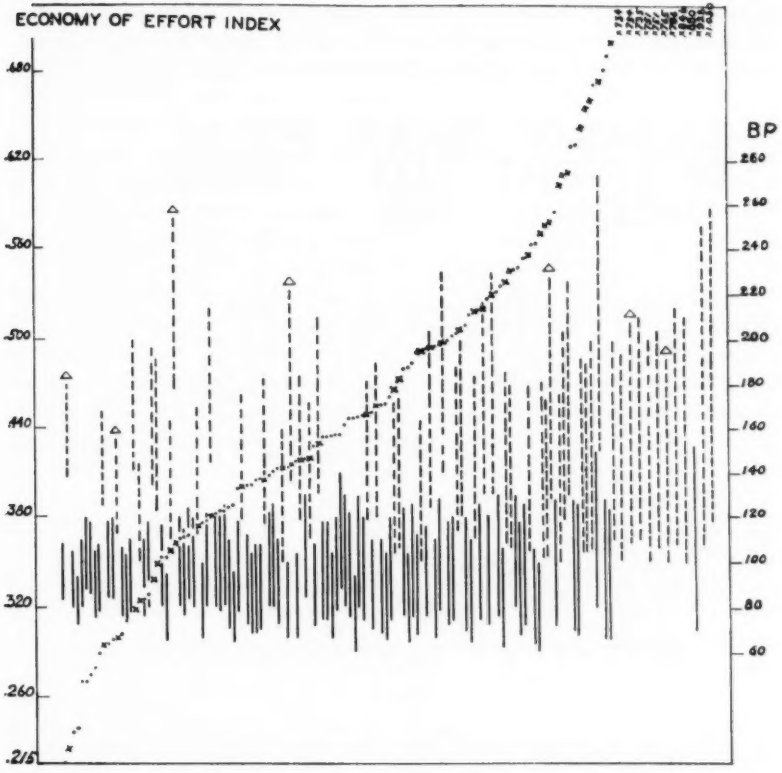

Fig. 3

heart rates—so that not only the volume of systolic discharge but also the economy with which it is expelled may vary sufficiently to be detected by this method. Since the index is so closely dependent upon the relationship between the pulse pressure and diastolic pressure it is not unreasonable to expect a wide range of the index in normal persons reflecting the normal variations in systolic discharge and peripheral resistance. However, if we choose the safer path of conservatism and regard the broad range of

the normal index as due to shortcomings in the principle of the method or its application we have at least established the normal values with which the index of pathological persons may be compared.

Discussion of data for hypertensive subjects. As shown in figure 3 the index expressing economy of effort for this group covers an even wider range from 0.220 to 1.030. Again it is seen that the index is greater in individuals with a large pulse pressure, a relatively low diastolic pressure or a combination of the two. None of the hypertensive subjects had an index below that of the lowest normal, many were considerably higher and of the twelve highest only one was a normal subject. Seventy

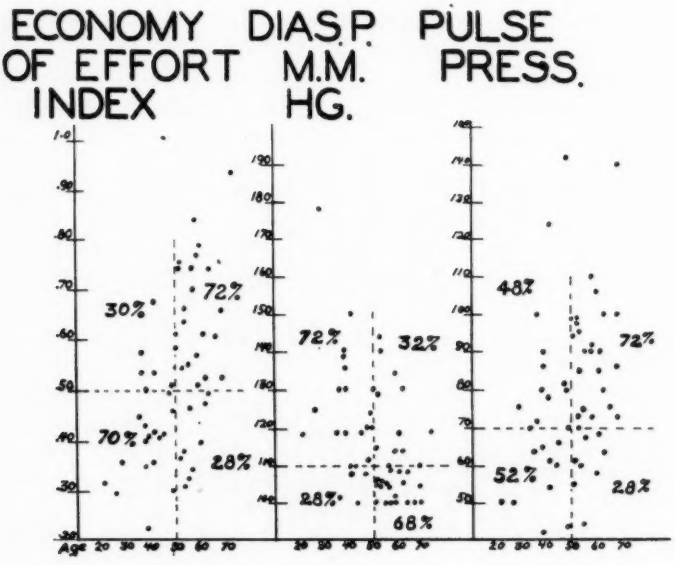

Fig. 4

per cent of the hypertensive subjects were found to have an index above 0.428, the median of the normal group. With few exceptions, the pulse pressure of the hypertensive individual was significantly larger than that of any normal subject. Nevertheless, this advantage was often offset by the high diastolic pressure as can be easily seen in comparing the pulse pressure and diastolic pressure of a normal and a hypertensive subject having similar values for the index of economy of effort. In other words, the hypertensive heart with a large pulse pressure often shows no better economy of effort than a normal heart with a small pulse pressure. It seems fair to interpret these data as showing that the hypertensive heart

utilizes its ejection energy just as economically, and oftentimes even more economically, as does the normal heart.

It is of interest to point out, as shown in figure 4, that in analyzing the data for the hypertensive group, those persons fifty years of age or more tended to have a larger index for economy of effort than did those persons below fifty years of age. Seventy per cent of the group fifty years of age or above had an index greater than 0.5, whereas only 30 per cent of the younger group had an index above 0.5. Further analysis of this group shows (fig. 4) that 68 per cent of the older group have a diastolic pressure lower than 110 mm. Hg, while only 28 per cent of the younger group of hypertensives are found to be below that diastolic level. Moreover, 72 per cent of the older group have a pulse pressure larger than 70 mm. Hg, whereas only 48 per cent of the younger hypertensives are found to be above that figure. In other words, the hypertensive persons fifty or more years old tend to a higher index of economy of effort by virtue of *both* a lower diastolic pressure and a larger pulse pressure.

In view of the lack of evidence for an increased systolic discharge in persons with hypertension (9) it is conceivable that these differences are due to *a*, decreased distensibility of the aorta, or *b*, decrease in the capacity of the aorta with the same systolic discharge. Since the latter, *b*, is not consistent with postmortem findings in hypertensive persons it would appear that increasing aortic sclerosis of advanced years serves the useful purpose not only of keeping the diastolic pressure relatively low but also of maintaining or improving the economy of ventricular effort.

SUMMARY AND CONCLUSIONS

1. A method, based upon a principle worked out from animal experiments by Wiggers and Katz, is suggested, by which the economy of effort during ejection of the normal human left ventricle can be expressed and compared with that of the left ventricle of a hypertensive subject.

2. Reconstruction of the ejection phase of the intraventricular pressure curve is accomplished using the subclavian pulse curve for contour and applying simultaneously obtained brachial artery pressures for the ordinate (pressure) values. The surface area of the curve above diastolic value divided by that beneath this area offers a quotient expressing the economy of ventricular effort during ejection.

3. Results for 81 normal individuals indicate a wide variation in the quotient (0.215–0.880) and it is greatest in those subjects having a comparatively large pulse pressure and a low diastolic pressure. This wide variation is explained by the normal variation in the relation of systolic discharge to peripheral resistance.

4. Results for 54 individuals with chronic hypertension show an even greater range of quotient (0.220–1.030) and the same relation to pulse

pressure and diastolic pressure is observed as was found in the normal individuals. A larger number of this group had a quotient above the median (0.428) of the normal group. The conclusion is reached that the left ventricle in hypertension maintains a quotient as good as or even better than that of the normal left ventricle by virtue of a large pulse pressure and in spite of an elevated diastolic pressure.

5. Evidence is given in support of the belief that decreased distensibility of the aorta when found in conjunction with hypertension particularly in older subjects, supplies a mechanism whereby the economy of effort during ejection remains normal or may actually become more favorable.

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PROPAGATION, AND EXTENSION OF EXCITATORY EFFECTS, OF THE NERVE ACTION POTENTIAL ACROSS NONRESPONDING INTERNODES

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According to the most generally accepted theory, propagation of the impulse along a nerve fiber is accomplished through reëxcitation by an extension of the potential arising in the active locus. Until recently the same theory has prevailed with regard to synaptic transmission of activity. At the present time, however, the view that synaptic transmission is accomplished by a chemical mechanism holds the ascendancy to the complete disregard, in some circles (e.g., Dale, 1934) of the possibility of any direct rôle of the electrical phenomenon. Moreover, accounts of the isolation from active nerve fibers of substances, such as acetylcholine (Binet and Minz, 1934) or acetylcholine-like substances (v. Muralt, 1937), which are considered to be the synaptic transmitters, imply that propagation in a nerve fiber, even, may be by a chemical mechanism, though doubt has been injected into this question by the failure to find acetylcholine in sensory fibers (Loewi and Hellauer, 1938).

In 1934 we (Erlanger and Blair) recorded potential extending ahead of the anodally blocked nerve impulse a distance subtending three internodes (3 to 4 mm.). The extension of potential ahead of an impulse blocked by cold or pressure has since been demonstrated by Hodgkin (1937), who showed, in addition, a parallel enhancement of the excitability of the nerve. We had shown independently that an impulse blocked at a node may so condition the fiber ahead, that a second impulse impinging on the block will stimulate the fiber beyond, presumably at the next node (Blair and Erlanger, 1936b). An isolated observation indicated that the impulse was conditioning the fiber across two inactive internodal segments. It was concluded that electrical transmission of impulses alone could furnish an explanation of these results. At that time the possibility was not considered by us that when a conditioning impulse restores the response of but one segment the blocking and the restimulation might be transpiring in one and the same node. Though this seems a very remote possibility, it nevertheless must be admitted, under the circumstances, that there was

but a single wholly unequivocal observation on which to base the conclusion that propagation of the nerve impulse can be accomplished by processes other than those requiring molecular contiguity. We now are reporting observations demonstrating beyond peradventure that a nerve impulse can *a*, condition a fiber, indeed *b*, that it can actually stimulate a fiber, across the length of at least one nonresponding internodal segment.

a. *Activation by temporal summation.* Since reporting the above-mentioned experiments we have succeeded in ascertaining conditions which permit one to demonstrate regularly the activation of two successive nodes through temporal summation of nerve impulses. As in the previous experiments (Blair and Erlanger, 1936b), the conducted action potential of a single fiber in a phalangeal preparation is led through an amplifier into the electron oscillograph and the nerve is polarized with anode at the proximal (earthed) lead and cathode a distance up the nerve (over four internodes) that puts it beyond the possibility of affecting the configuration of the record. Then, in the present experiments, the common electrode (i.e., the proximal lead and the anode) is, by trial, placed in such a location on the nerve that the threshold blocking current for node *d* (fig. 1) is lower than that for node *c*, and the strength of the blocking current is set at the level that blocks node *c*. One may then be certain that the block at *d* will effectually stop transmission of any single impulse started in internode *3*. In order to attain this condition very nice adjustments of the position of the electrode and of the strength of the polarizing current are necessary, but through the use of this procedure it is possible to demonstrate with any phalangeal preparation having a fiber of outstanding excitability the full restoration of a spike when two successive nodes have been thus blocked by anodal polarization.

The data from an illustrative experiment may be cited. By shifting slightly the position of the common electrode, the extreme range of polarization strength was found 1, within which both node *c* and node *d* could be blocked while still retaining temporal summation that would overcome the block, and 2, within which the blocking threshold of node *d* was lower than that of node *c*. In three positions meeting these conditions polarizing strengths (readings on the potential divider) of 446, 458 and 436 blocked at node *d*: the normal negative phase of the conducted spike (see fig. 1, A), made up primarily of the responses of segments *1*, *2*, *3* and *4*, was converted into one consisting of the responses of segments *1*, *2* and *3* (fig. 1, B). And when this action potential was followed by another after an interval of about 3 msec., the latter was conducted the length of the fiber as may be seen in B, *1-N*. Increasing the current strengths to 448, 467 and 450, or 0.5, 2.0 and 3.2 per cent, respectively, in the three trials mentioned above, blocked the initial impulse at node *c* also, so that the record consisted of a spike made by segments *1* and *2* only (fig. 1, C);

yet the full spike (1-N) was restored by this antecedent conditioning, though blocked, action potential. It was not possible to find in the same general locality a position that would permit a greater percentile increase in the strength of the blocking current that still was compatible with the end in view.

The remote contingency that the action potential is blocked and also reactivates the fiber in node *c*, and that the action potential thus started in segment *3* is stronger than the normal one and consequently succeeds

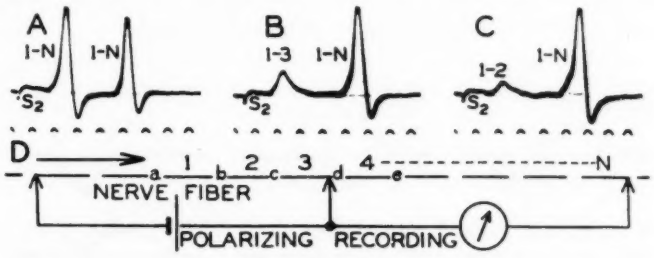

Fig. 1. Records from an experiment demonstrating a lowering of threshold by a blocked nerve impulse extending ahead along the fiber a distance subtending certainly one, and probably two, internodal segments. The interval between conditioning and conditioned responses is about 3 msec.

A. Anodal polarization short of blocking intensity. Both spikes complete; the 2nd, being in the relatively refractory period of the first, is the lower.

B. The conditioning impulse is blocked at node *d*; and the record is made by the potentials of segments *1*, *2* and *3*. The conditioning but blocked spike so lowers the threshold at node *d* that the 2nd action potential runs the length of the fiber.

C. The blocking current is increased so as to block not only at *d*, but also at *c* so that initially only the responses of segments 1 and 2 record. The conditioning, but blocked, spike lowers the threshold both at *c* and *d* and the second action potential then courses the length of the fiber.

S_2 is the artifact of the shock starting the second spike on its way; the artifact (not shown) of the 1st shock is 3 msec. to the left of S_2.

Time: 1000 cycles per sec.

D. A diagram showing a relation between the anode and the fiber segments such as would yield the action potentials portrayed.

in overcoming the block at *d*, is cared for (a) by the fact that the height of the conducted spike of segment *3*, as indicated by the position of the notch on the second spike (B, 1-N), is no greater than that of segment *3* when its spike is unconducted (B, 1–3), and (b) by the fact that the block at *d* is being maintained by a strength of anodal polarization that is definitely in excess of the one required to block there.

Considered in conjunction with our previous papers the present experiments, therefore, demonstrate that the potential extending ahead of the active locus in a fiber lowers the threshold ahead a distance subtended

certainly by the length of one internodal segment, and in all probability by the length of two internodal segments.

b. *Restimulation of a fiber by its action potential.* To demonstrate actual restimulation of a fiber by its action potential across an inactive stretch, the impulse is blocked at one node while the excitability at the next node is being enhanced. These conditions are supplied by polarizing the phalangeal preparation through electrodes separated by less than the assumed internodal distance with the cathode at the proximal lead (P) into the amplifier and the anode central to it (see fig. 2). An electrode separation of about 1 mm. has been found to be satisfactory.

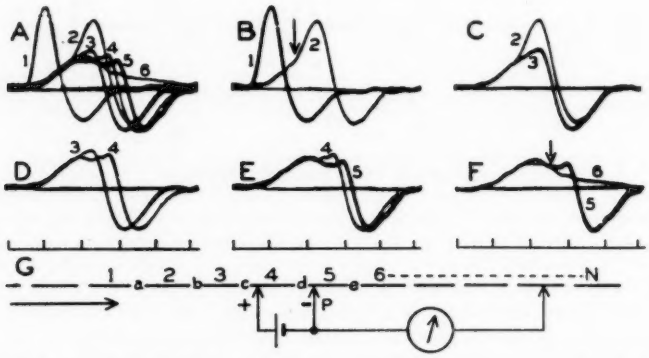

Fig. 2. Six records (*1–6*) from an experiment demonstrating restimulation of a fiber by its action potential across the length of one, possibly two, nonresponding internodal segments. The fiber is polarized as indicated in G.

In A the starts of the six records are superimposed. In B to F, successive pairs are similarly superimposed so that details obscured in A may be seen.

C shows, in the spontaneous change between 2 and 3, the loss of an all-or-nothing contribution to the spike with maintained conduction; F, the loss of another segment response with block. For further details see text.

Time intervals are 0.5 msec.

G. A diagram showing relations between electrode positions and fiber segments such as would yield the results portrayed in this figure.

Previous observations have shown that cathodal polarization of the fiber through the proximal lead produces a series of changes in the configuration of the conducted action potential (see Erlanger and Blair, 1934, fig. 3, A and E) which are quite comparable to those displayed by records *3*, *4*, *5* and *6* of figure 2 here. As the current strength is increased the negative (upward directed) phase of the diphasic action potential broadens, lowers and develops notches. At a critical current strength the impulse is blocked, the sign of block being the abrupt disappearance both of the part of the negative phase beyond a notch (arrow in fig. 2, F) and of the entire positive phase.

In the previous series of observations the fiber was polarized anodally directly through the lead electrode. The effect (see fig. 3, A, B and C there) was to heighten and broaden the negative phase of the action potential and to develop notches on it. When the impulse was blocked the part of the spike above some one of the notches disappeared and again the action potential became monophasic. The action potential becomes monophasic at this stage both in this and in the preceding case because block in the region of the proximal lead prevents the impulse from proceeding into the domain of the distal lead. In the present series the anode, as has been said, is placed about 1 mm. proximal to the lead. In this position it will not exert as much effect on the height of the spike as when it is located at the lead, but its ability to block will be unimpaired and will depend primarily upon the proximity of the electrode to a node. The combined effects exerted by the anode and by the cathode when, as in the present experiments, they are in close proximity to each other, can be inferred from the two known separate effects.

We may now consider the results of a typical experiment demonstrating that the action potential can proceed across nonresponding internodal segments of a fiber. When the position and separation of the two polarizing electrodes have been happily chosen (and considerable manipulation may be required to attain this end) the action potential responds to an increase in the strength of the polarizing current by changes in configuration such as are shown by the selected records reproduced in figure 2. At first the action potential broadens, develops a notch on its ascending limb, and decreases somewhat in height. This change in configuration is not unexpected since, as explained above, it is the resultant of the effects produced through the anode and through the cathode as they affect the proximal lead. At a critical and steady polarization level the successive pictures in the present case change without any regular sequence between three quite definite types. The action potential records sometimes as *2* (first type), sometimes as *3*. This change obviously is due to a loss in area in the negative phase, primarily. Since there are no intermediate stages the loss must be the result of occasional failure of a significant, and more or less constant, length of the fiber to contribute to the negative (the upward directed) phase of the spike. And since the higher of the spikes (*2*) has mainly the characteristics of one derived from anodally polarized nerve, whereas, as will be seen, the lower one is converted into the third type through a series of changes characteristic of those produced by cathodal polarization, it is concluded that the block that converts the first into the second type develops at the anode. *The record, however, remains diphasic.* It follows that, despite the block, the impulse must still be reaching the distal (the grid) lead. It appears, therefore, that though one internode has failed to respond, the impulse nevertheless is reaching the end of the fiber.

Records *3*, *4* and *5* all belong to one, the second, type. We are convinced of this because the series of records obtained includes every gradation between the extremes shown in the figure. Moreover, they are, as has been seen, exactly comparable with the changes in configuration the spike undergoes when the proximal lead from a fiber is exposed to the influence of the cathode only, at strengths of current short of those required to block.

There are no gradations between records of the second type and record *6*. The latter is of the third type; it is the result *a*, of the loss of another quantum of area, namely, the part back of the notch indicated by the arrow, and *b*, of the development of monophasicity. Unquestionably, the alteration is determined by the establishment of a block under the cathode which now effectually prevents the impulse from reaching a place in the interlead region where it would reverse the direction of the potential difference acting on the leads.

It has been stated that all of the pictures ranging between *2* and *6*, inclusive, could be recorded at one setting of the polarizing current. In explanation of this variability under apparently constant conditions it may be assumed that the two polarizing electrodes were occupying positions with respect to the nodes through which they were exerting their maximum effects such that each was at its blocking threshold. The variability of the pictures, then, would be the expression of spontaneous changes in the excitability of the fiber (Blair and Erlanger, 1933), the block developing sometimes at one of the nodes, sometimes at the other, sometimes at both and sometimes at neither.

Those familiar with the pictures obtained when two or more fibers with the same excitability are being stimulated at their threshold (see Blair and Erlanger, 1933) might be inclined to regard records *2* to *6* as the portrayal of that state of affairs. It is highly improbable, however, that three fibers with spikes of such diverse shapes would conduct at *exactly* the same rates. Moreover we have accepted for this type of experiment only preparations containing a fiber of such outstanding excitability that there was no possibility that any other fiber could have responded to a stimulus of the strength employed. For instance, in the case used here for illustration it was necessary to increase the stimulus 32 per cent above the threshold of the most irritable fiber in order to reach the threshold of the next fiber in point of irritability.

The results obtained in the illustrative experiment may now be considered in terms of a diagram (fig. 2, G) which is believed to portray the situation actually obtaining. The impulse coursing in the direction of the arrow is recorded diphasically. At the critical polarization strength the impulse sometimes is blocked at node *c* by the anelectrotonus, some-

times at both node *c* by the anelectrotonus and node *d* by the catelectrotonus. Whenever it is blocked at *c* alone the spike loses a quantum of its area, the response of segment *4*; but since the record remains diphasic, it follows that node *d* has been stimulated, and presumably by currents produced in the last active segment, no. *3*, and eddying through node *c*. Whenever node *d* then blocks (cathodally) the spike loses another quantum of its area, that contributed by segment *5*; it is a small quantum, however, since the fiber is cathodally polarized at the locus concerned; the record at the same time becomes monophasic since the potential extending from the last active segment, still no. *3*, is not high enough at *e* to restimulate the fiber there.

It is improbable, however, that the situation is quite so simple. When a block is established at *c* the current determined by segment *3* would eddy ahead and reinitiate the impulse at node *d*. The action potential from segment *5* acting through *d* would add its effect to that from segment *3* and would activate segment *4*, also. It therefore follows that not until the threshold of segment *4* has risen to such a level that the combined potentials from segments *3* and *5* fail to stimulate segment *4*, would the contribution of this segment be lost. On this basis *3* is the picture that is made up of the contributions of segments *1*, *2* and *3*, of segment *5*, activated probably in the manner indicated above in the case of segment *4*, and of segments *6* to *N* conducting toward the distal lead from *e*. In other words, the current emanating from node *c* now apparently is stimulating the fiber at node *e*, or across two inactive internodal segments. But however interpreted, the pictures signify that conduction is maintained although at least one segment is failing to respond.

The safety factor of the action potential as an electrical stimulus. On the assumption that propagation is electrical, various estimates have been made of the excess height of the spike over the liminal height required for propagation. H. A. Blair (1934) estimates on the basis of latencies of nerve responses reported by Blair and Erlanger (1933) that the spike of high irritability fibers is about twice, of low irritability fibers about four times, that required for propagation. Rushton (1937) calculates that the spike has three times the threshold for conduction, but considers this value much too low. Hodgkin (1938) finds that the just subconducted local response of an unmyelinated crustacean nerve fiber has about one-fifth the height of the spike. This experimentally derived value indicates that in crustacean nerve the spike has about five times the strength required for propagation.

In order to obtain a measure of the safety factor in vertebrate nerve we have proceeded in another manner. If, as assumed in the foregoing, conduction is electrical, then if we further assume *a*, that the strength of

the spike is proportional to its recorded amplitude, and b, that the threshold in terms of the spike is proportional to the threshold in terms of a condenser discharge, a relation is obtained which may be expressed as:

$$\frac{\text{Recorded spike height}}{\text{Shock threshold}} \times \text{Constant} = \text{Spike strength in terms of threshold.}$$

The value of the constant may be determined by anodally polarizing the phalangeal nerve, one with a fiber of outstanding excitability, to a point just short of block for this easily identifiable fiber, while recording the spike impinging on, and determining the threshold of, the depressed locus.[1] Since that spike is just adequate for stimulation, the strength of the spike in terms of threshold is equal to 1. All other factors being known, the value of the constant may then be calculated. On ceasing to polarize, the normal spike height and the threshold of the same locus can again be determined, and since the constant is known, the strength of the normal spike may be calculated. In any given nerve with anodal polarization the agreement between observations on different loci is quite satisfactory, but in different nerves the value of the spike in terms of conduction threshold have ranged in fifteen determinations on seven nerves from 1.02 in manifestly depressed nerve, to 2.46.

When the constant is determined through a block produced by cathodal polarization less reproducible results are obtained, principally because of a progressive change in threshold. In five determinations on different nerves, the value for the spike ranged from 0.74 in a fiber in which conduction did not return following the polarization, to 3.94. This last result, which is the highest of the entire series, indicates that 25 per cent of the spike might serve for propagation. Because of the greater reproducibility and of the theoretical consideration mentioned below we accept the maximum value determined by anodal polarization as most nearly representing the safety factor for the most irritable fiber of our preparation, indicating that about 40 per cent of the spike is necessary for excitation.

That there are certain possibilities of experimental error in the use of this method of estimating the stimulating value of the action potential is realized. 1. The record of the spike is made not only by the potential impinging on the depressed locus, but also by a contribution from the depressed locus; therefore the observed change in spike height due to polarization is somewhat greater than that of the effective spike height. 2. Though the polarizing and stimulating circuits are one, determinations of excitability occasionally have been complicated by stimulation away from the depressed locus. This complication is manifested by an increase in the apparent latency following the testing shock, and when this occurred the experiment has been

[1] To be certain that we were measuring the threshold of the fiber producing the spike recorded, we have employed refractoriness to the conducted spike as the index to excitation at the lead.

discarded. 3. Cathodal polarization probably increases accommodation and the change in threshold to a short shock is probably not an adequate index to the change in threshold to a spike. With anodal polarization more satisfactory threshold readings should be obtained with testing shocks of constant duration because in our experience there is little accommodation in normal nerve, even with rheobasic stimulation (Blair and Erlanger, 1936a), and anodal polarization opposes accommodation.

DISCUSSION. The present experiments then have shown *a*, that an action potential blocked by anodal polarization lowers the threshold in the fiber beyond the block for a distance subtended certainly by one, probably by two, internodal segments, and *b*, that under the conditions supplied, the action potential so blocked actually can restimulate the fiber across the length of one, and possibly of two, inactive internodal segments. In addition, the experiments indicate *c*, that the action potential of this preparation has a stimulating value approximating 2.5 thresholds. Considered from the most conservative standpoint the experiments demonstrate that progression of the nerve impulse can be determined by an electrical mechanism under conditions that preclude the intervention of any process dependent upon molecular contiguity.[2]

If the action potential can do these things across an inactive stretch of nerve fiber 1 to 2 mm. long, it certainly is justifiable to conclude that it can do them also across a synapse and that it will unless the synapse includes a device for preventing current spread, and there are no reasons for believing that it does. The liberation of "chemical transmitters" then becomes an adjunct to transmission at the synapse, one which, at least under certain circumstances, can initiate impulses of itself, but which is not essential to the process of restimulation. In other words, "the chemical transmitter" might play a rôle comparable to that of the cathodal polarization employed by us in the experiments demonstrating restimulation of a fiber by its action potential.

The experiments have shown that it is possible to supply conditions under which saltatory progression will occur along a fiber, and we now have to consider *a*, whether saltation can occur under normal conditions, and *b*, how far removed from the normal was the nerve under the conditions we supplied.

a. It is known that an externally applied potential falls to about half value every 2 mm. of nerve length and we have published evidence (Erlanger and Blair, 1934) indicating that the extrinsic potential of the active locus declines very roughly to half value per segment length (assumed to be 1.25 mm.). Since the action potential has a stimulating value of about

[2] Since the submission of this manuscript we have seen the paper by Hodgkin (J. Physiol., **94**: 560, 1939) showing that the velocity of conduction in a crab fiber is affected by the outside electrical resistance.

2.5 thresholds there is every reason for believing that saltatory progression will occur where there are segments of normal length and provided there is no continuous progression that is faster.

b. In our experiments it was necessary to polarize cathodally the node beyond the one anodally blocked. We do not know definitely the degree to which the cathodal polarization lowered the threshold there. But since continued cathodal polarization may not lower the threshold to half the normal value immediately under the cathode, even, it is safe to say that the threshold at node *e* (fig. 2), located, as it was, some distance beyond the cathode, was somewhat higher than that. Since the distance was that of two internodal segments, it seems fair to conclude that the ratio of stimulus to threshold in our experiments did not differ materially from that obtaining normally.

The rôle of the medullary sheath has been the subject of much speculation (see Gerard, 1931). It has a high resistance and undoubtedly acts as an insulator. That it is an insulator is indicated by the fact that activity of a part of the medullated fibers of a nerve does not affect appreciably the excitability of neighboring inactive fibers in the same nerve (Blair and Erlanger, 1932), whereas nonmedullated crustacean fibers that are on the verge of spontaneous discharge may be stimulated by the action potential in other fibers if the two are brought close together in parallel (Jasper and Monnier, 1938).

The interruptions in the medullary sheath might subserve two ends either or both of which might be responsible for the spacings of nodes. *a.* Since it is known that exchanges between axons and surrounding medium take place at nodes (Erlanger and Blair, 1938) and that all medullated fibers are provided with nodes (Bielschowsky, 1928), it is possible that the spacings are determined, at least in part, by nutritive needs of the fiber. But, disregarding the reserve factor as it may affect nerves of different diameters, the small fibers with their relatively larger surfaces might be expected to require for nutritive ends fewer communications with the surrounding medium than the large fibers, yet the situation is just the reverse of this. It should be added, though, that we have no information regarding the width of nodes relative to fiber diameter. *b.* In the light of present knowledge the suggestion is ventured that a potent factor influencing the spacing of nodes as the segments are laid down and subsequently altered (see Speidel, 1933), might be the electrical phenomenon itself. For reasons which need not be presented now, we do not regard as convincing, the evidence which led Speidel to conclude that myelin is deposited before developing fibers begin to function.[3]

[3] Speidel informs us that the outgrowing myelin-emergent fibers he followed might have been branches of a fiber, other branches of which had reached their terminations, and therefore might have been conveying impulses.

Now that it has been demonstrated that conduction can be, and probably is, saltatory it may be justifiable to speculate, also, regarding the relation segmentation might bear to conduction rate. Zotterman (1937) has confirmed in mammals the results we obtained in frogs which led us to the conclusion that the velocity varies as the square of the diameter of the fiber. When, however, he plots data derived from Key and Retzius, he finds that segment length varies as fiber diameter, and says that "a linear relation between the rate of conduction and the diameter of the nerve fibre would strengthen" the view that progression of the nerve impulse might be saltatory. But, since the velocity of propagation actually varies as the square of the diameter, "one has to assume," he goes on to say, "that the rate of transmission across the Ranvier's nodes varies as the squares of the diameter of the fibres."

Now, if certain assumptions are made, which to us seem to be justifiable, it can be shown that the square relationship of velocity to fiber size is not incompatible with the saltatory progression. If the configuration of the spike is determined by the electrical network of the nerve (see fig. 39 in Erlanger and Gasser, 1937), instead of being a record of the local process itself, and if the resistance of the axis cylinder is the most potent factor in that network, then varying this resistance would vary the time functions of the spike without otherwise varying its configuration. If, then, we further assume that for any fiber a constant fraction of the height of the spike is needed for stimulation, the time, t, required to stimulate a segment would vary as the core resistance or as $\frac{l}{d^2}$, where l is the length of a segment and d the diameter of the fiber. But, as Zotterman shows, l varies as d. Therefore t varies as $\frac{l}{d}$. Now the time, T, required to traverse a given distance varies as $n \times t$ or as $\frac{n}{d}$, where n is the number of nodes involved. But n also varies as $\frac{l}{d}$, whence T varies as $\frac{l}{d^2}$. And since the velocity, V, varies as $\frac{l}{t}$ we have V varies as d^2.

SUMMARY

1. Two successive nodes of Ranvier depressed by anodal polarization can be so conditioned by a blocked nerve impulse that a second impulse will pass both nodes.

2. The extrinsic potential from a nerve impulse blocked at the anode of a constant current can be made to stimulate the fiber at the cathode, thus reinitiating the impulse across an inactive gap of one or two internodes.

3. A method is described of determining experimentally the stimulating

value of the action potential; the value accepted amounts to about 2.5 thresholds.

4. The significance of the findings is discussed relative to *a*, temporal summation and impulse transfer at a synapse, and *b*, saltatory progression of the nerve impulse.

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THE EFFECT OF PHOSPHOLIPID INGESTION UPON THE GAS EXCHANGE IN MAN[1]

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During the course of some researches to determine whether or not sprue patients can tolerate lecithin better than neutral fat, 60 grams of mixed soya bean phospholipids were fed to normal subjects by stomach tube and the respiratory quotient, urinary phosphate and nitrogen, blood sugar, serum inorganic phosphate and phospholipid determined. The surprising observation was made that the R.Q., under the conditions of the experiment, quickly dropped to a minimum in a half-hour and then rose to a maximum as high as unity in two to four hours. To determine to what extent this phenomenon was due to the phosphate, to the fatty acid, or to the phospholipid as a molecular unit, equivalent amounts of disodium glycerophosphate, of olive oil, and of mixtures of disodium glycerophosphate and olive oil were fed to subjects and the same determinations made.

LITERATURE. The effect upon the R.Q. of feeding lecithin or its non-lipid constituents has received but little attention.

Gregg (1932) obtained no change in R.Q. after feeding lecithin to dogs. Yet Gregg as well as Kovaliova (1912) obtained increases in R.Q. after injecting that phospholipid. This rise in R.Q. might be related to the observations of Izar and Constantino (1929) and of Jost (1931). The former reported a significant rise in blood sugar after the injection of lecithin and the latter a rise in the production of sugar when cephalin was added to the blood of a perfused liver.

Page and Young (1932) and Yriant (1931), working with phlorhizined and depancreatized dogs respectively, could not show this production of sugar from phospholipid.

Kovaliova (1912) and McCann and Hannon (1923) obtained a rapid lowering in R.Q. after the injection and ingestion respectively of glycerophosphates. Ablin (1925, 1926, 1929) and Schmutzer (1928) showed that when phosphate is fed with carbohydrate the rise in R.Q. is delayed.

[1] A preliminary report of this paper was presented before the American Society of Biological Chemists, April 1, 1938, at Baltimore, Maryland.

[2] Anna H. Hanes Fellow in Medicine.

The effect of fat feeding upon the gas exchange has received more attention, especially from Murlin and his students. Until 1933 Murlin believed, as did Lusk and many others, that all R.Q. values of less than 0.700 were due to errors in their determination or of some other effect not the direct result of metabolism. However, at this time work was done in his laboratory (Hawley et al., 1932, 1933) which showed quite conclusively that pigs and humans with a high tolerance for fat will give R.Q. values lower than 0.700 after ingesting fat and that the R.Q. will rise 0.04 to 0.06 unit above the basal level about seven hours after the fat meal. Later Murlin (1936) assumed that in R.Q. values below 0.707 all the non-protein CO_2 is from fat combustion and that the extra O_2 absorbed is concerned in partial oxidation of fat. He was not convinced, however, that the fat had been converted to carbohydrate, but pointed out that the fatty acids may be oxidized along the chain to some other product.

In most studies on R.Q. after the ingestion of fats no observations were made before one and a half or two hours after the test meal. As will be shown in the present work, however, the R.Q. is almost at its lowest level after one-half hour when small amounts of fat are fed. Gregg obtained such an effect shortly after feeding fat to rats (1931) and considered it as due to a transient acidosis and ketonuria. A similar observation was made by Wilder, Boothby and Beeler (1923) who were studying the metabolism of a diabetic. These authors made no effort to explain such an early drop.

Methods.[3] The respiratory quotient was determined by collecting the exhaled air for five minutes in a Douglas bag and analyzing it in a Haldane apparatus. Blood and urinary phosphates were determined by the method of Fiske and Subbarow (1925), phospholipid by the method of Boyd (1931), urinary nitrogen by the Kjeldahl method, blood sugar by Benedict's method (1931), and blood CO_2 content by the Van Slyke volumetric apparatus.

Experimental. In our earlier experments the phospholipid, as obtained from the Glidden Co.,[4] contained about 30 per cent of oil. This was removed by dissolving in petroleum ether and precipitating with acetone, the procedure being carried out several times. Later the Glidden Co. sent the material fat-free. The major portion of soya bean phospholipid is cephalin, the rest mainly lecithin. (Nottbohm and Mayer, 1932.)

In preparation for the experiments 60 to 65 grams of the purified preparation were emulsified in five volumes of water. The subjects were allowed no food or tobacco after 6:30 the evening preceding the experiment. At

[3] The gas analyses and urinary nitrogen determinations were made by Miss Hanes Clement.

[4] The soya bean phospholipids used in this study were generously supplied by The Glidden Company, of Chicago, Illinois.

TABLE 1

Metabolic and biochemical effects of the ingestion of 60 grams of phospholipid

SUBJECT	PERIOD	NON-PROTEIN GAS EXCHANGE (LITERS PER HOUR)			MGM. PER CENT SERUM PHOSPHOLIPID	MGM. PER CENT SERUM INORGANIC PHOSPHORUS	MGM. PER HOUR URINARY INORGANIC PHOSPHORUS
		CO_2	O_2	R.Q.			
	hrs.						
J. B. A.	Basal	7.02	9.75	0.720	165	3.8	17
	½	7.24	10.15	0.714			
	1	7.49	9.55	0.785	187	4.2	13
	2	7.72	9.10	0.849	213	4.9	27
	3	7.96	8.35	0.954	195	6.3	63
	4	7.52	9.02	0.834	168		63
	5	9.44	10.85	0.870	161		32
	6	9.64	11.55	0.835		5.4	11
P. E.	Basal	6.00	7.41	0.810	165	4.2	40
	½	5.13	6.98	0.735			55
	1	6.39	8.31	0.770	165	4.3	70
	2	6.49	8.25	0.787	180	4.8	84
	3	6.47	8.03	0.806	187	4.8	85
	4	7.34	8.74	0.840	124	5.1	73
	5	6.59	8.51	0.774			73
E. D.	Basal	9.79	12.30	0.796		4.21	20
	½	9.44	12.50	0.755		4.13	10
	1	9.24	12.15	0.784		4.35	3
	2	9.51	11.70	0.814		4.50	35
	3	9.24	12.10	0.764		5.06	22
	4	10.44	13.40	0.780		4.71	17
	5	9.01	11.85	0.760		4.60	71
T. W. O.	Basal	9.39	12.73	0.738			
	½	8.86	11.58	0.765			
	1	8.52	11.26	0.756			
	2	8.84	10.76	0.822			
	3	9.19	10.45	0.880			
	4	9.44	11.45	0.824			
	5	8.77	11.02	0.795			
L. J.	Basal	3.95	5.59	0.707	158	4.17	20
	½	5.25	7.41	0.709		4.51	27
	1	5.14	6.97	0.738	159	5.08	31
	2	5.69	7.40	0.770	200	5.40	103
	3	5.32	7.12	0.747	183	5.95	111
	4	5.64	7.35	0.767	170	6.12	190
L. M.	Basal	5.70	7.55	0.736			
	1	5.84	8.14	0.718			
	2	6.60	8.07	0.818			
	3	6.06	7.80	0.777			
	4	6.36	8.47	0.751			

TABLE 2

Metabolic and biochemical effects of the ingestion of 60 grams of phospholipid (incomplete)

SUBJECT	PERIOD	NON-PROTEIN GAS EXCHANGE (LITERS PER HOUR)			MGM. PER CENT SERUM PHOSPHOLIPID	MGM. PER CENT SERUM INORGANIC PHOSPHORUS	MGM. PER HOUR URINARY INORGANIC PHOSPHORUS
		CO_2	O_2	R.Q.			
	hrs.						
C. R. D.*	Basal	Overbreathed			161		
	2	12.08	14.74	0.820	172		
	3	11.93	12.84	0.930	183		
	4	13.43	12.74	1.055	194		
	5	10.23	11.64	0.880	153		
	6	8.43	11.84	0.712	161		
J. F.	Basal	Overbreathed					18
	½	7.70	9.52	0.810	185	3.28	22
	1	8.73	10.08	0.866	205	4.20	25
	2	7.88	9.22	0.855		4.80	47
	3	8.00	9.50	0.843	213	5.00	87
	4	8.18	8.83	0.927	179	5.20	95
	5	7.68	8.47	0.907	179	5.50	112
	6	7.72	9.97	0.774			99
R. L. E.	Basal	Overbreathed			223	3.00	15
	½	7.07	7.70	0.920		3.12	17
	2	6.82	7.77	0.878	216	4.20	21
	3	7.63	7.41	1.040	223	4.75	37
	4	6.51	5.95	1.094	245	5.60	38
	5	6.43	5.78	1.113	245	5.80	55
	6	7.55	8.12	0.930	238	5.92	47
C. D.	Basal	Overbreathed			176		13.1
	½	9.06	11.06	0.818			9.28
	1	8.48	10.28	0.826	176		22.7
	2	8.93	9.74	0.917	176		57.8
	3	9.54	10.19	0.935	227		61.1
	4	8.23	9.39	0.878	193		64.0
	5	8.05	10.13	0.795	224		60.0
R. T.	Basal	Faulty analysis					
	½	6.79	8.75	0.777			
	1	7.73	9.75	0.793			
	2	7.95	9.70	0.820			
	3	7.25	8.60	0.843			
	4	8.62	9.58	0.900			
	5	8.26	10.37	0.795			

* This subject received 100 grams.

5:30 or 6:00 o'clock on the morning of the experiment they were asked to void as completely as possible and were given a glass of water. At about 7:30 they were wheeled on a chair or stretcher to the room in which the experiments were conducted. After a short rest the basal R.Q. was determined. In spite of the precaution of taking the subjects into the room the preceding day and training them in breathing into the bag, several over-breathed for the basal test. After the air had been collected for the basal R.Q. blood was collected for the determination of blood sugar, serum

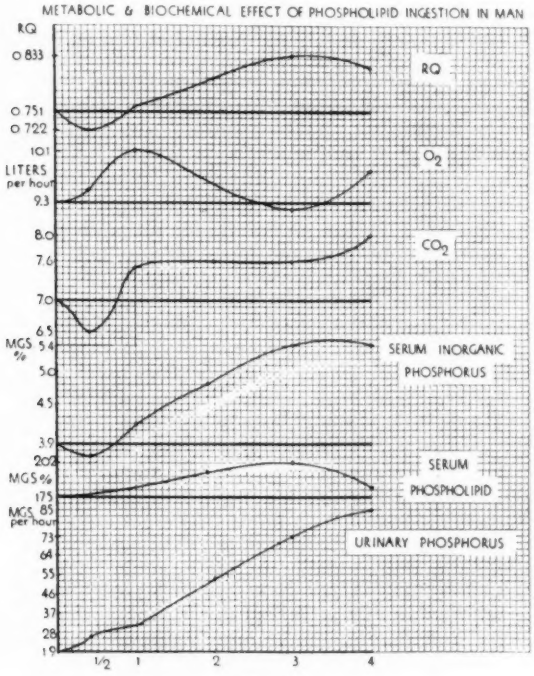

Chart 1

inorganic phosphate, and serum fats, and, in a few cases, blood was collected under oil for CO_2 content. After the blood was drawn the patient voided as completely as possible. Inorganic phosphate and total nitrogen were determined on the urine obtained.

The test meal was then given, the phospholipid being passed by stomach tube. In only one case did the subject become nauseated. This was C. R. D. who, because of his size, was given 100 grams of phospholipid. After that experience all subjects were given 60 grams with no untoward

effect. Experiments were continued from 4 to 6 hours, the air, blood, and urine collections being made in that order.

The results of the phospholipid experiments are given in tables 1 and 2 and chart 1. The blood sugar also was determined in many cases, but it did not change significantly. The R.Q. and gas values in the chart are only for those experiments in which trustworthy determinations were made in every period. Table 2, however, shows that in those experiments which were not complete there was a similar rise in R.Q.

TABLE 3

Metabolic and biological effects of the ingestion of 16 grams of disodium glycerophosphate

SUBJECT	PERIOD	NON-PROTEIN GAS EXCHANGE (LITER PER HOUR)			MGM. PER CENT SERUM INORGANIC PHOSPHORUS	MGM. PER HOUR URINARY INORGANIC PHOSPHORUS
		CO_2	O_2	R.Q.		
	hrs.					
I. W. B.	Basal			0.765		
	½			0.680		
	1			0.716		
	2			0.775		
	3			0.779		
	4			0.762		
J. P.	Basal	9.74	10.21	0.954	2.98	23.1
	½	7.09	9.98	0.710	3.35	24.4
	1	7.59	10.00	0.759	3.35	47.2
	2	8.57	10.33	0.830	3.57	60.0
	3	7.35	9.53	0.772	3.16	71.0
	4	7.45	9.93	0.750	3.16	75.0
C. B.	Basal	8.71	10.96	0.785	4.63	7.7
	½	7.58	9.63	0.796	4.63	10.9
	1¼	7.55	10.12	0.746	5.40	26.2
	2	7.92	10.62	0.745	5.71	37.4
	3	8.18	10.34	0.754	5.71	40.0
	4¾	8.34	10.34	0.769		43.0

Since the results of the phospholipid experiments were so different from those reported in the literature for fat, it was thought that the effects might be due to the phosphate. Accordingly, three subjects were given 16 grams of disodium glycerophosphate. In the first experiment only the total R.Q. was determined, but the last two patients were studied as completely as after phospholipid feeding. The results of these experiments are given in table 3.

Three subjects were given a mixture of 45 grams of olive oil with 16 grams of disodium glycerophosphate to determine if the effects of the

phospholipid were due to the molecule as such. The results are tabulated in table 4.

Finally, the effects of 45 grams of olive oil alone were determined on three subjects. The results are summarized in table 5.

DISCUSSION. The mean values of all the determinations are compared in chart 2. In order to have a basis for comparison all basal values were reduced to zero and the unit changes plotted. The R.Q. changes appear to show no interrelationship, those after phospholipid appearing to be

TABLE 4

Metabolic and biochemical effects of the ingestion of 45 grams of olive oil and 16 grams of disodium glycerophosphate

SUBJECT	PERIOD	NON-PROTEIN GAS EXCHANGE (LITERS PER HOUR)			MGM. PER CENT SERUM INORGANIC PHOSPHORUS	MGM. PER HOUR URINARY INORGANIC PHOSPHORUS	MGM. PER CENT SERUM PHOSPHOLIPID
		CO_2	O_2	R.Q.			
	hrs.						
J. L.	Basal	10.44	12.07	0.866	3.75	42	198
	½	8.60	11.70	0.735	4.22	36	191
	1	9.35	10.35	0.904	5.00	53	176
	2	9.60	10.75	0.893	5.40	66	187
	3	9.73	11.62	0.837	5.26	68	187
	4	10.26	11.62	0.890	5.08	59	194
E. D.	Basal	8.76	11.20	0.782	4.16	12	175
	½	7.88	11.48	0.686	4.21	27	
	1	10.06	13.75	0.732	4.40	17	175
	2	9.29	12.37	0.751	4.95	30	187
	3	9.32	12.27	0.780	5.15	32	187
	4	10.16	12.45	0.816	5.15	69	180
	5	9.60	12.77	0.751	5.00	115	187
C. D.	Basal	8.07	9.15	0.882	4.30		194
	½	8.61	10.66	0.808	4.55	13.4	
	1	8.18	10.06	0.805	4.55	13.4	194
	2	8.11	9.78	0.830	5.13	28	194
	3	8.13	9.58	0.848	5.72	61	194
	4	8.39	10.51	0.798	5.72	60	194

quite different from those after its constituents. However, there is a similarity in the oxygen and carbon dioxide changes after oil plus phosphate to those after phospholipid and a dissimilarity of these changes to those after phosphate or oil alone. The changes in serum and urine inorganic phosphorus make it quite evident that the absorption of fat increases absorption of phosphate and that this effect is greatest after phospholipid. Verzar and Laszt (1934) have presented evidence indicating that glycerol phosphate increases the absorption of fat, although Irwin,

Weber and Steenbock (1936) could not confirm it. An increase of 25 per cent in serum phospholipid after the ingestion of 60 grams of phospholipid, but none after equivalent amounts of olive oil or oil plus phosphate, presents the possibility that some of the complex compound may have been absorbed unchanged.

There are several possible explanations of the high R.Q. values after phospholipid ingestion. One of these is that it is an acidosis effect. However, the elevated R.Q. corresponds to a decrease in oxygen consumption

TABLE 5

Metabolic and biochemical effects of the ingestion of 45 grams of olive oil

SUBJECT	PERIOD	NON-PROTEIN GAS EXCHANGE (LITERS PER HOUR)			MGM. PER CENT SERUM INORGANIC PHOSPHORUS	MGM. PER HOUR URINARY INORGANIC PHOSPHORUS	MGM. PER CENT SERUM PHOSPHOLIPID
		CO_2	O_2	R.Q.			
	hrs.						
B. L.	Basal	8.15	11.55	0.706	3.64	28	225
	½	8.13	11.68	0.696	3.46	17	233
	1	8.19	11.03	0.743	3.33	16	215
	2	8.35	10.80	0.773	3.68	19	222
	3	8.23	11.21	0.735	3.64	26	229
	4	8.83	11.85	0.745	3.59	29	
R. B.	Basal	6.76	7.67	0.882	4.21	47	
	½	4.92	6.07	0.811	4.04	42	
	2	7.09	9.37	0.757	4.12	36	
	3	8.18	9.66	0.846	4.45	39	
	4	7.26	8.77	0.827	4.45	47	
E. D.	Basal	9.79	12.30	0.796	4.21	20	187
	½	9.44	12.50	0.755	4.13	9.7	
	1	9.24	12.15	0.784	4.35	12	187
	2	9.51	11.70	0.814	4.50	15	191
	3	9.24	12.10	0.764	5.06	18	191
	4	10.44	13.40	0.780	4.71	36	202
	5	9.01	11.85	0.760	4.60	44	202

after an early increase, and not to a blowing off of carbon dioxide. Furthermore, the changes in the gas exchange after phosphate plus oil resemble closely the changes after phospholipid but are very different from the changes after either of these alone. It is thus unlikely that the effect is one of acidosis.

A second possibility is that feeding phospholipid stimulates carbohydrate metabolism. However, it hardly seems reasonable that the ingestion of phospholipid should depress fat metabolism and stimulate the mobilization and combustion of glycogen stores.

It is possible that the fatty acids of the ingested phospholipid undergo a partial oxidation in the intestinal mucosa during absorption. This would account for the observed increase in oxygen absorption. These partially oxidized fatty acids may then be carried to the tissues, where the oxidation is completed, causing a reduction in the amount of oxygen absorbed.

The possibility that fats are oxidized in a stepwise manner has been proposed from time to time. Very recently Werthessen (1937), by feeding

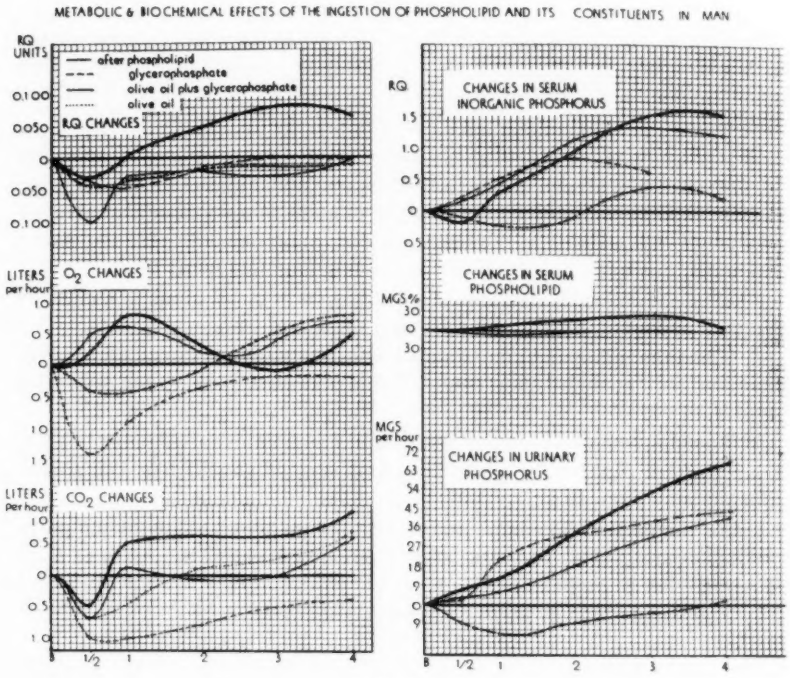

Chart 2

rats at a certain time of the day and no other, obtained R.Q. values ranging from 0.27 to 1.70. He explained these results by a chain reaction system in which the fats are first converted to a complex carbohydrate and then to a simple one of the glucose type.

It is probable that only a portion of the ingested fat goes through these stages during absorption, the major part being absorbed as fat and stored. Any condition, therefore, which would cause a larger part of the absorbed fat to be oxidized in the manner described would show evidence of the

stepwise oxidation in the gas exchange. The presence of phosphate would appear to be such a condition since phosphate and fat apparently aid each other reciprocally in absorption, and the gas exchange after feeding the two together presents a different picture from that after either alone. Unpublished work in this laboratory shows that there is a lowering of blood and urinary inorganic phosphate during the absorption of fat, which is further evidence that phosphate is a factor in the absorption and metabolism of the lipids.

SUMMARY AND CONCLUSIONS

1. It was found that after feeding 60 grams of soya bean phospholipids to humans the non-protein R.Q. fell during the first half-hour and then rose, reaching a maximum considerably above the basal value in three to four hours. It was shown that this was mainly an oxygen effect.

2. That this effect was not entirely one of the phospholipid molecule as such was shown by similar effects upon the oxygen absorption and carbon dioxide elimination of a mixture of olive oil and disodium glycerophosphate equivalent in amount to the phospholipid. In the latter condition, however, the R.Q. did not rise above the basal level.

3. That the effect is not due to the phosphate or oil alone is shown by the very different results after feeding these substances separately.

4. The increase in serum inorganic phosphate was greatest after phospholipid feeding, slightly less after oil plus phosphate, and much less after phosphate alone.

5. The elimination of urinary phosphate was greatest after phospholipid, less but more rapid after phosphate, and somewhat still less and slower after oil plus phosphate.

6. An increase of 25 mgm. per cent in serum phospholipid after phospholipid feeding was not found after feeding equivalent amounts of olive oil or oil plus phosphate.

7. The significance of these findings is discussed.

The author is deeply obligated to Dr. Wm. A. Perlzweig and Dr. Frederic M. Hanes for advice and criticism.

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STUDIES ON THE SECRETION OF BILE

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Having available a method (1) which permits a quantitative study of the volume output of bile by the liver, we have made a study of certain factors which affect the composition of bile.

METHODS. In our method the continuous application of a slight negative pressure to the tubing draining the biliary passages has eliminated fluctuations in bile flow due to kinks, mucus and precipitate which may temporarily retard or obstruct the flow, a difficulty observed by Whipple and his collaborators (2) when they employed the Rous-McMaster method (3). This latter method may be used, however, if adequate attention is given to its imperfections; this is shown by a careful perusal of the studies of Whipple and his associates on the metabolism of bile salts in the dog, by our own experience with the method and by the fact that our outputs of bile salt under basal conditions check well with those of Whipple. The amount of suction applied was slightly more than the amount required to overcome the resistance of the collecting apparatus, which varied from eight to sixteen inches of water. Two other requisites for quantitative results regarding volume output and bile salt are the appetite of the animal and the establishment of a basal control state before the introduction of any new procedure. Both of these requisites are mentioned because they have frequently been ignored; their importance has been emphasized by Whipple and his associates and we have found them to be essential. In addition our animals were prepared so that bile could be returned slowly to the duodenum after feeding at any desired rate. Only animals in excellent condition and manifesting no infection of the biliary passages were used; an hepatitis is immediately reflected by a decrease in bile salt output.

Bile acids were determined as cholates by Reinhold and Wilson's modification of the Gregory-Pascoe method (4). This procedure detects only cholic acid; however, since taurocholic acid is the principal if not the only bile acid in dog's bile, the method was entirely satisfactory for our purpose.

[1] This work was assisted by a contribution from the E. L. Dawes Research fund.

Duplicates and the method in the hands of three different persons check within 3 per cent. *Cholesterol* was determined by the method of Elman and Tausig (5). *Total pigment* was assayed by a method reported previously (6). In those experiments in which specific determinations for *bilirubin* were made the van den Bergh reaction as modified by Gibson and Goodrich (7) was used.

Diet. Following recovery from the operation the animals were maintained on a weighed standard diet containing 9 per cent carbohydrate (cereal), 12 per cent protein (meat and milk) and 6 per cent fat, supplemented daily by 10 cc. of cod liver oil and 3 grams of dried yeast. Since the caloric need of bile fistula animals is rather high, seventy-five calories per kilo were given daily to maintain pre-operative weight. Except during special tests, the dogs were fed three times daily (7–7–10 hr. intervals usually) and all of the bile secreted one day was returned to the intestine the following day; *this statement must be remembered to interpret properly certain results.*

The bile was returned following meals usually at the rate of 1 cc. per minute. The dogs weighed from 8 to 15 kilos and all maintained their weight.

RESULTS. *Experimental variations.* Six dogs were studied over a period of one month to ascertain the spontaneous experimental variations in volume and cholic acid output when the dogs were fed the diet referred to above and a measured portion of the bile was returned to the duodenum post-cibum at a rate of 1 cc. per minute. The detailed results for a period of five consecutive days on one of the dogs are shown in table 1. The results for five consecutive days on another dog are shown in figure 1. In this figure the results with and without the return of bile to the intestine are contrasted. *The daily variation in volume and cholic acid output did not deviate more than ± 8 per cent from the mean daily values.*

In two of the six dogs, the cholesterol and pigment output on the basal diet with the return of bile was followed for one month in addition to the volume and cholic acid output. The cholesterol and pigment output appeared to manifest an unrelated cyclic variation when the volume and cholate output remained more or less constant. The cycles lasted for from four to six days and caused a variation in cholesterol and pigment output of from ± 15 to ± 20 per cent respectively. No attempt was made to establish this point by an extensive study. Yet, it is clear that a ± 20 per cent deviation from the mean in cholesterol and pigment output must be taken into account in interpreting the effects of various procedures on the biliary constituents. Although in various test-periods of from three to five days' duration, or before and after the introduction of some new procedure the volume and cholic acid output did not vary more than ± 8 per cent, the pigment and cholesterol output would vary as

much as ± 20 per cent from one period to another during two or more months of study. (This statement pertains to results obtained on eight dogs.) (Fig. 1.)

When the animals were placed on the basal diet and no bile was returned the volume output of bile did not vary more than ± 3 per cent, but the

TABLE 1

Period and daily output showing experimental variations

Dog II-3. Maximum bile salt return: output given in milligrams, body weight 8 kilos, and daily ration given in three equal portions followed by slow return of bile.

DATE (DECEMBER)	PERIOD	VOLUME			CHOLATES				CHOLESTEROL				PIGMENT			
						Output				Output				Output		
		In cc.	Out cc.	Per cent ±	In mgm.	Out mgm., 1 cc.	Total mgm.	Per cent ±	In	Mgm. per cent	Total mgm.	Per cent ±	In	Mgm. per cent	Total mgm.	Per cent ±
1	Day—7 hours	110	122	3.9	2640	20.7	2525	5.5	19.6	3.0	3.70	12.9	39.6	30.2	36.8	22.0
	Evening—7 hours	110	144	5.9	2640	21.4	3080	3.7		3.7	5.40	5.5	39.6	24.7	35.6	24.6
	Night—10 hours	110	162	1.2	2640	17.5	2835	4.0		2.3	3.70	35.1	39.6	21.8	35.4	26.0
	24 hours		428	0	7920		8440	+3.2			12.80	—14.9	118.8		107.8	—21.6
2	Day—7 hours	110	126	0.8	2640	20.0	2520	5.7		3.48	4.4	3.6	39.6	31.2	39.3	16.7
	Evening—7 hours	110	134	1.5	2640	23.4	3140	5.5	19.6	3.82	5.10	0.4	39.6	25.6	34.3	27.3
	Night—10 hours	110	158	3.7	2640	17.0	2700	0.9		3.24	5.10	10.5	39.6	30.3	48.0	0.4
	24 hours		418	—2.3	7920		8360	—2.5			14.60	—2.9	118.8		121.6	—11.5
3	Day—7 hours	125	138	8.7	2640	20.7	2860	7.0		3.18	4.40	3.6	40.4	35.4	48.8	3.4
	Evening—7 hours	125	136	0.0	2640	20.0	2720	8.6	21.4	3.73	5.06	0.8	40.4	33.6	45.7	3.2
	Night—10 hours	125	178	8.5	2640	17.7	3160	15.9		2.95	5.25	7.9	40.4	29.7	52.8	10.4
	24 hours		452	+5.6	7920		8740	+2.0			14.73	—2.6	121.2		147.3	+7.1
4	Day—7 hours	125	126	0.6	2640	20.9	2620	1.9		2.64	3.33	45.0	40.4	39.5	62.2	30.8
	Evening—7 hours	125	128	5.9	2640	21.1	2700	9.2	21.4	4.0	5.12	0.0	40.4	41.0	62.5	11.2
	Night—10 hours	125	162	1.2	2640	18.8	3025	10.9		4.8	7.78	36.5	40.4	38.3	49.8	4.2
	24 hours		416	—1.9	7920		8345	—2.6			16.23	+7.7	121.2		164.5	+19.7
5	Day—7 hours	125	124	2.4	2640	22.8	2830	6.1		4.26	5.30	22.3	40.4	39.5	48.8	3.4
	Evening—7 hours	125	138	1.5	2640	23.4	3230	8.7	21.4	3.53	4.88	4.6	40.4	31.2	43.1	6.9
	Night—10 hours	125	162	1.2	2640	17.9	2900	6.5		4.14	6.70	17.6	40.4	32.8	53.2	11.3
	24 hours		424	—0.9	7920		8960	+4.6			16.88	+12.2	121.2		145.1	+5.5
Ave.	Day—7 hours		127				2672				4.25				47.2	
	Evening—7 hours		136				2975				5.12				47.2	
	Night—10 hours		164				2724				5.70				47.8	
	24 hours		428				8570				15.04				137.5	

cholic acid output varied ± 8 per cent from "period to period" during two or three months of study. (We say from "period to period" because we have never kept our dogs off bile for more than a five-day period.)

We have also used the closed-fistula method of Rous and McMaster (3)

for the study of cholic acid output. We find that if proper precautions are taken, the cholic acid output is about as consistent as when the "suction method" is employed. The volume output of bile is from 50 to 100 per cent more and the daily variations are less when the "suction method" is used. Thus any obstruction or resistance to the flow of bile produced by the collecting system is first reflected in the volume output of bile.

The secretory response to a meal. The output of fluid, cholic acid, cholesterol and pigment by hourly periods after feeding a meal with and without the return of bile. Experiments were performed on six dogs to determine the

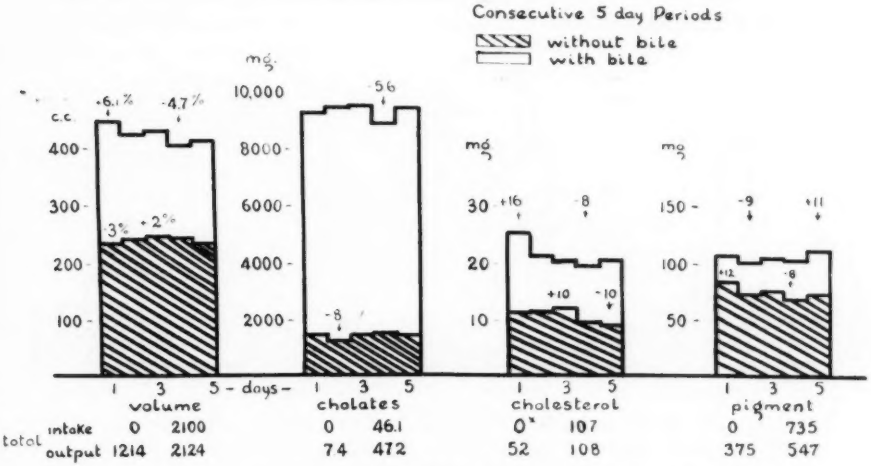

Fig. 1. This figure shows the output of bile, cholates, cholesterol and pigment in a dog on a standard diet with and without the return of bile for a five day period. When the animal was on the diet but bile was not returned the output of cholates for the period was 7.4 grams, or 1.47 gram per day; when 46.1 grams, or 9.2 per day, of cholates in the form of the animal's own bile were returned, the output of cholates was 47.2 grams, or 9.4 grams per day.

effect of feeding a meal, which consisted of the diet described above fed every eight hours or three times during the twenty-four. After the animals had been on this regime for several days, bile was collected hourly and analyzed. A minimum of three such experiments were performed on each dog with and without the return of bile by the "suction method." In all animals the response was characteristic in every test, so that the results may be illustrated by a single figure (fig. 2).

When no bile is returned to the intestine after feeding the meal, a slight but significant increase in volume occurs which reaches a maximum during the 3rd or 4th hour post-cibum. The constituents of the bile do not share in

this increase. During the first two post-cibal hours the output of cholic acid is only slightly increased or is essentially unchanged. (Compare the cross-hatched figures in fig. 2 with the previous eighth-hour post-cibal control.) The slight increase in cholesterol during the first hour or two could be the result of a "flushing out" type of process due to the diminished hepatic secretory activity toward the end of the preceding eight-hour period. The output of pigment is similarly increased but to a slightly greater extent than cholesterol. The striking observation is that when no bile is returned and the animal is fed every eight hours, cholic acid manufacture and output are remarkably constant, a fact previously observed by Smith, Groth and Whipple (2) when the dog was fed once daily without the return of bile.

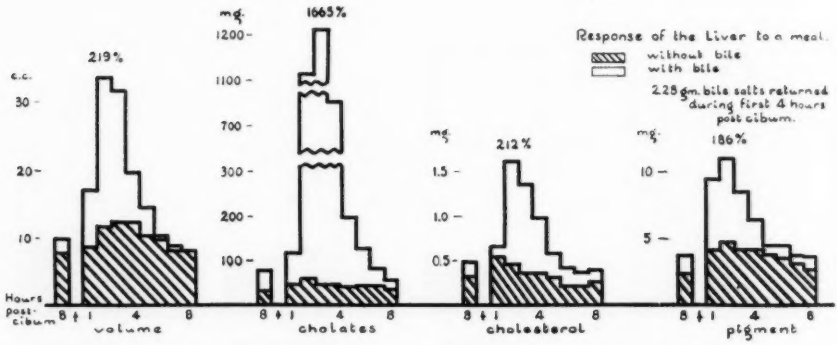

Fig. 2. This figure shows the hourly output of bile, cholates, cholesterol and pigment after the feeding of a meal with and without the return of bile. The animal was fed every 8 hours for several days and the first single column represents the 8th hour secretion preceding the 8-hour test-period. When bile was returned, the dog's own bile containing 2.28 grams of cholates was introduced into the duodenum during the first 4 hours post cibum.

The picture changes very decidedly *when bile is returned.* (Most of the bile secreted during the previous day was divided into 3 portions and returned at each eight-hour feeding period during the first 4 hours post-cibum.) In the particular test illustrated by figure 2 the total amount of cholic acid returned in 4 hours amounted to 2.28 grams. On comparing the curves for *volume, cholesterol and pigment,* it is noted that the magnitude of the maximum percentage increase (fig. 2) is essentially the same, about 200 per cent. The concentration of cholesterol and pigment does not change significantly; thus, the increased elimination of these constituents *apparently* occurs as a result of increased volume of fluid secreted. (Hourly *phosphatase* determinations made during one such test on 2 dogs indicate that this constituent of bile, also, shares the same relation to the volume

of fluid secreted.) With the *cholic acid output*, however, a different relationship is obtained; the concentration of cholic acid is doubled and sometimes tripled at a time when the fluid output is undergoing a decrease. The result is a marked increase in bile salt output, the maximum occurring during the third hour when the rate of flow of fluid, while still elevated, is diminishing.

These observations on cholic acid elimination are in contrast to those of others (8, 9) who have studied the elimination of bile salts injected intravenously in acute experiments or given orally by stomach tube to one bile-fistula dog. The generalization from such experiments, chiefly acute experiments, is that the usual range of variation in the concentration of bile acids as secreted by the liver is relatively slight, and an increase in bile acid output is attained primarily through the medium of an increase in volume of the bile. If this were always the case, we should have obtained a maximum increase in hourly bile acid output of around 200 per cent (comparable to the other constituents) instead of the observed 1,600 per cent increase.

The foregoing tests on the secretory response of the liver to a meal with the return of bile to the duodenum were devised primarily to imitate as closely as possible the "normal" conditions. The gall bladder empties its concentrated bile along with hepatic bile in from 2 to 4 hours after a meal; it was for this reason that the greater part of the bile secreted (we only kept out a portion for analysis) during an 8-hour period in response to a meal and the return of bile was returned during a period of 4 hours. If we had desired to imitate the conditions of cholecystectomized animals, then we would have returned to the intestine that amount of bile secreted each hour and at the rate it was secreted. Such a procedure could probably delay by one or two hours the time of occurrence of the peaks. One might think that to duplicate the effect of gall-bladder bile, the hepatic bile should have been concentrated from 4 to 8 times; this was not done because it is the total bile salt in the bile administered and not the fluid or water that affects bile output by the liver, since water may be absorbed from the intestine without affecting bile volume output (1). It should also be pointed out that the 7.92 grams of cholic acid in table 1 and the 9.2 grams of cholic acid in figure 1 made the enterohepatic circuit but once daily, according to the conditions of the experiments, whereas in actual life the cholic acid may make several such circuits each day (*vide infra*).

Data regarding the output of cholic acid, cholesterol and bile pigment in the bile when known amounts of the substances are introduced into the intestine after a standard meal. The dogs were stabilized on the basal diet without the return of bile for a period of from 3 to 5 days (suction method). The dogs then received by the intestine a bile of known composition for

a period of from 3 to 5 days, after which the bile was withdrawn. It was found, as has been observed by others (10) that 10 or 12 hours after the administration of the last bile, the cholic acid output had returned usually to precontrol levels.

Cholic acid output. When no bile was returned the output of cholic acid varied in the different dogs (eight) from 1.2 to 1.6 grams daily. This value represented the endogenous production plus the effects of the diet, assuming that the two are additive. When practically all the bile secreted was returned, the output of cholic acid ranged from 6.5 to 9 grams in the different dogs. When no bile was returned, the cholic acid output in the different dogs ranged from 100 to 170 mgm. per kilo body weight per day. These values check well with those obtained by Smith,

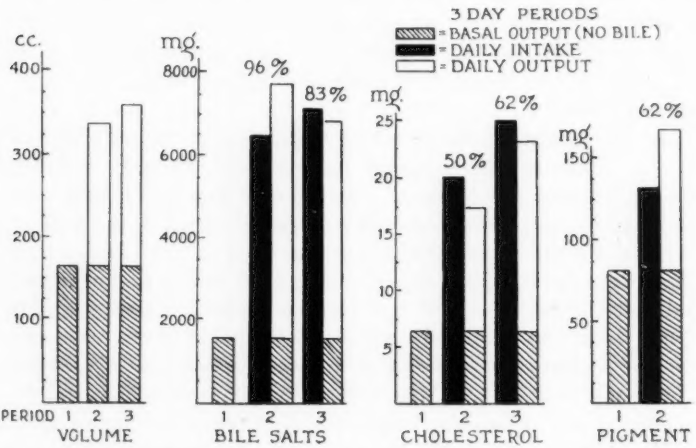

Fig. 3. This figure shows the "recovery" of cholates, cholesterol and bile pigment when the dog's own bile is returned to the duodenum in different amounts.

Groth and Whipple (2), if one considers that our diet contained 12 per cent protein and theirs 7.25 per cent, and our bile acid is expressed as cholic acid and theirs as taurocholic acid.

In figure 3 are shown the results on one of three dogs in which bile was returned at two different levels. When bile was administered containing 6.5 grams of cholic acid, or an amount which approximated the daily output of this animal when it received all the bile it excreted, 96 per cent of the cholic acid administered was recovered. (This figure is determined by adding the daily output of cholic acid on the diet without bile to the cholic acid administered, the total yielding the cholic acid theoretically available for excretion.) When the cholic acid administered was raised to 7.2 grams the animal developed diarrhea and the per cent "recovered" was

diminished. The other dogs did not develop diarrhea at that level of intake and the average per cent of cholic acid recovered was 90 per cent. At a higher level of intake they developed diarrhea. The average per cent recovered as calculated either from the amount returned or the total available for excretion in the six dogs referred to in the experiments illustrated by figure 1 and table 1 was also approximately 90 per cent. Some of our results on the recovery of orally administered bile salts in dogs prepared by the method of Rous and McMaster are shown in table 2. Ox-bile salts in an amount containing 2.5 or 1.5 grams of cholic acid were administered daily with the diet. It is to be noted that the loss or gain of cholic acid eliminated daily in the bile varied from −30 to +28 per cent; this range of variation is greater than in the dogs studied by the suction method. Yet, when the average recovery of the cholic acid fed is calculated, the recovery is found to be 91 per cent. Thus, approximately a 10 per cent loss of the cholic acid available for elimination was found in both groups of animals. In table 2, however, it is to be noted that only about 85 per cent of the cholic acid given orally was recovered in the bile.

At what site in the enterohepatic circuit this loss of 10 or 15 per cent of the cholic acid occurs is uncertain. It may be destroyed by bacteria, lost in the feces, or destroyed in the liver. (See Sobotka, *Physiological Chemistry of the Bile.*) Yet, if some loss did not occur, bile salts would accumulate and intermittently provoke diarrhea. The daily loss must balance the daily synthesis. This balance is probably accomplished as follows: When practically all of the bile is returned daily, the dog on our basal diet keeps from 7 to 9 grams of cholic acid in circulation. Whipple and Smith state 7 or 8 grams. But, it should be recalled that under the conditions of our experiment, the 7 to 9 grams was permitted to circulate only once daily, and that our results on the hourly output of cholic acid show that practically all of the cholic acid introduced into the intestine during a three or four-hour period reappears in the bile in from 6 to 8 hours and that the first bile salt given appears in the bile within a half-hour. Hence, under normal conditions of digestion, a given portion or all of the bile salt may traverse the enterohepatic circuit three, four, or five times daily. If 10 per cent is lost during each circuit, then of the 9 grams that made the first circuit only 6.7 grams would make the fourth circuit. If the original 9 grams of cholic acid made only two circuits daily, then the total loss would be 1.6 grams, and 1.6 grams of bile salt (the average is actually about 1.4 grams on our diet) is approximately that amount of bile salt excreted on the diet alone. *Thus, if from 7 to 9 grams of cholic acid traversed the enterohepatic circuit three times daily, the total loss would compensate for that formed endogenously and from the diet, and a more or less constant output would be maintained.*

This concept is supported by the results of Kocour and Ivy (1) in that

TABLE 2

Showing the variations in volume and cholic acid output under controlled conditions on a basal diet without the return of bile and with the administration of cholic acid as ox-bile salts orally by the Rous and McMaster method at various 3 to 5 day periods over 2 to 4 months in various dogs

This table also shows the recovery of orally administered cholic acid from the bile.

"C"—control; "T"—treated with bile salt; dogs fed twice daily. When bile salt was given, the dose was divided and given with the meals.

DOG	WEIGHT	VOL. OUTPUT, CC.			CHOLIC ACID OUTPUT, MGM.			RECOVERY OF CHOLIC ACID FED			RECOVERY OF TOTAL CHOLIC ACID AVAILABLE		
		C	T	Per cent gain	C'	T'	Per cent gain	Mgm. fed, A	T' − C'	Per cent gain, loss	C' + A	Total output, T'	Per cent gain, loss
	kgm.												
IV 1 A	9	135	207	55	1333	3193	139	2500	1860	−26	3833	3193	−17
IV 2 B	8.2	134	205	53	1283	2660	107	2500	1377	−45	3783	2660	−30
II 4 C	11.2	125	217	116	1160	3583	206	2500	2423	−3	3660	3583	−2
II 7 D	11.4	75	162	74	1476	3090	109	2500	1614	−36	3976	3090	−22
E 1	8.4	133	198	49	1320	3501	165	2500	2181	−13	3820	3501	−8
E 1	8.4	138	198	43	1354	3501	159	2500	2147	−14	3854	3501	−9
F 2	12	134	207	55	1244	4802	286	2500	3558	+42	3744	4802	+28
F 2	12	137	215	57	1307	4300	229	2500	2993	+20	3807	4300	+13
IV 3	8	123	167	36	1356	2761	103	1500	1405	−6	2856	2761	−3
		134	165	23	1346	2475	84	1500	1129	−25	2846	2475	−13
IV 4	10.9	115	144	25	1605	2863	78	1500	1258	−15	3105	2863	−7
IV 4	10.9	140	159	13	1558	3140	101	1500	1582	+5	3058	3140	+2
		159	227	42	1701	3405	100	1500	1704	+14	3201	3405	+6
IV 1	9	128	171	33	1280	2257	76	1500	977	−35	2780	2257	−18
		133	168	26	1236	2357	90	1500	1121	−25	2736	2357	−13
IV 8	11	109	170	56	1257	2044	62	1500	787	−48	2757	2044	−26
		117	197	68	1304	2965	127	1500	1661	+11	2804	2965	+5
		117	167	43	1304	2555	95	1500	1251	−17	2804	2555	−8
IV 5	12	96	123	38	1536	2450	59	1500	914	−39	3036	2450	−19
		103	138	34	1339	2547	90	1500	1206	−20	2839	2547	−10
		102	132	34	1540	2779	80	1500	1239	−18	3040	2779	−8
1	8.4	109	159	46	1370	2931	114	1500	1561	+4	2870	2931	+2
		156	193	24	1537	2606	69	1500	1069	−29	3037	2606	−13
		133	170	27	1155	2643	128	1500	1488		2655	2643	0
2	12.0	135	148	9	1285	2655	106	1500	1370	−9	2785	2655	−5
		132	157	19	1203	2037	69	1500	834	−47	2703	2037	−24
Ave.	2.5	126	201	60	1309	3580	173	2500	2271	−9	3809	3580	−6
	1.5	124	164	32	1384	2637	90	1500	1253	−16	2884	2637	−8.6

when bile and food were given every six hours during the twenty-four, the six-hour output of bile was very constant over many days. This would not have occurred if bile salt had accumulated. In their experiments most of the bile salt was circulated four times daily. When the animal is given his daily ration in three meals with the return of the bile secreted during the preceding eight-hour-period evidence of accumulated action does not occur, as was noted by Whipple and Smith (10) when they fed their animals and returned bile by stomach tube once daily. In the eight-hour-period experiments the 10 per cent loss of cholic acid with each circuit plus that in the bile removed for chemical analysis was enough to prevent accumulation and to maintain a balance.

Cholesterol. The output of cholesterol in the bile was always increased when bile was returned to the intestine (figs. 1, 2 and 3). Since the output on the diet without the return of bile varied in the different dogs (11) from 5 to 18 mgm. per day (0.5 to 1.4 mgm. per kilo body weight per day) and was subject to a ± 15 per cent variation from one experimental period to another, one should expect rather wide variations in the per cent increase when bile is returned to the intestine (12). When bile was returned, the output of cholesterol in the bile was usually increased only about 50 per cent, if the control output was high; if the control output was low, then the output was increased 200 or 250 per cent (figs. 1, 2 and 3). No strict correlation existed between the increase in volume output and cholesterol output. In a series of tests on three dogs the average per cent volume increase on the return of a measured quantity of bile was 80 per cent and the increase in cholesterol output was 140 per cent. In five experiments on two dogs and three experiments on one dog, the average increase in volume output was 87, 70, and 60 per cent and in cholesterol 218, 104, and 261 respectively; or the average increase in volume was 71 per cent and in cholesterol output 171 per cent.

Occasionally very consistent outputs of cholesterol would be obtained on the diet alone over a five day period; for example: 10 mgm. per day would be excreted, and then when bile containing 20 mgm. per day was returned the dog would put out 20 or 21 mgm. per day, to return to 10 or 14 mgm. per day when the return of bile was stopped (fig. 1). Or, it appeared as if about 60 per cent of the cholesterol in the bile returned to the intestine was absorbed and excreted. In the dogs represented by table 1, it appeared as if 50 per cent of the cholesterol in the returned bile was absorbed and excreted. In the various experiments performed, it appeared as if from 33 to 66 per cent of the cholesterol introduced into the intestine was absorbed and excreted again into the bile.

To obtain more direct evidence regarding the effect of stimulation of volume-output of bile on the output of cholesterol in the bile, we gave 4 dogs enterally 2.5 grams daily of cholic acid in the form of ox-bile salts,

which contained no cholesterol or bile pigment. In each dog after a control period of three days on the diet without the return of bile, the bile salts were given each day for a three-day period, which was followed again by a three-day control period. The average increase in volume output of bile in the 4 dogs when bile salt was administered amounted to 74 per cent; the cholesterol output was raised from an average of 10 mgm. to 21 mgm. per day or an average increase of 110 per cent. The increase in volume output in each dog was 57, 68, 74, and 116 per cent and in cholesterol output was 69, 69, 167, and 350 per cent respectively. Again a relation, although not a strict one, between volume and cholesterol output is observed. Wright and Whipple (11) found that when bile salt was added to the usual bile feeding cholesterol output by the liver was increased.

The effect of 50 mgm. per kilo of sodium dehydrocholate given *intravenously* on cholesterol output was determined by Dr. William Bradley on five dogs whose body weight averaged 10.7 kilos and on which an "acute" biliary fistula was made. The averaged results follow: *Control hour*, vol., 2.19 cc.; cholesterol concentration, 17.2 mgm. per 100 cc.; total cholesterol output, 0.261 mgm. *1st hour after bile salt*, vol., 16.4 cc.; cholesterol con., 4.29 mgm.; total cholesterol output, 0.532 mgm. *2nd hour after*, vol., 5.51 cc.; cholesterol con., 6.41 mgm.; total cholesterol output, 0.28 mgm. *3rd hour after*, vol. 3.03 cc.; cholesterol con., 9.07 mgm.; total cholesterol output, 0.264 mgm. Thus, the intravenously administered bile salt augmented cholesterol output during the hour of increased volume output, and the cholesterol output returned to the control level when the bile volume output returned to the control level. This evidence is intended to illustrate the importance of considering the effect of increased secretion of fluid by the liver in interpreting the effect of various procedures, including dietary factors, on the output of cholesterol in the bile (11, 12).

There is no need to labor the point by the submission of other evidence of this sort, because such evidence does not answer the specific question pertaining to what portion of the cholesterol in the bile is absorbed from the intestine and excreted by the liver. Such evidence only establishes the point that more cholesterol is excreted in the bile when bile or bile salt is present in the intestine. One cannot answer the question even by giving pure bile salt enterally to fasting dogs because the presence of bile salt or acid in the intestine may increase the absorption of the cholesterol or sterols excreted by the intestinal mucosa in fasting. The only way the question can be answered is by labeling the cholesterol. However, there is no reason to doubt that biliary cholesterol is absorbed to some extent by the intestinal mucosa, because it appears to be established by the literature that cholesterol is absorbed by the intestine and that the bile salts facilitate its absorption. But, as Wright and Whipple (11) have shown, relatively little of the cholesterol available for absorption from dietary sources is excreted in the bile of the dog.

Bile pigment. When bile was not returned to the intestine, the basal output of pigment on the diet ranged from 40 to 110 mgm. per day (5–8 mgm. per kilo body wt.). When bile was returned the pigment output ranged from 100 to 160 mgm. per day. (Bile pigment was consistently absent from the urine of our dogs.)

In the experiments referred to before and designed to ascertain the *apparent* recovery of bile pigment introduced as bile into the intestine, the average recovery, assuming the basal output to be constant, was about

TABLE 3

Typical data showing the effect of ox-bile salts given orally on bile pigment output; averages for three day controls and test periods by the Rous and McMaster method

"C"—control; "T"—test-period

DOG NO.	VOLUME, CC. PER 24 HOURS			TOTAL PIGMENT OUTPUT, MGM. PER 24 HOURS			CHOLIC ACID ORALLY
	C	T	Per cent change	C	T	Per cent change	
							grams
IV-1	143	167	+17	94	122	+30	1.5
IV-1	142	171	+20	68	66	−3	1.5
IV-1	142	207	+46	68	63	−7	2.5
IV-2	122	205	+68	63	61	−3	2.5
IV-3	115	144	+25	95	97	+2	1.5
IV-4	125	217	+74	73	89	+22	2.5
1	139	198	+42	109	94	−16	5*
1	109	159	+46	88	95	+8	1.5
1	133	170	+28	53	65	+22	1.5
1	143	198	+45	69	50	−27	2.5
2	131	185	+41	105	109	+4	5*
2	131	207	+58	105	111	+5	2.5
Average				82	85		
Average of 16 tests on 4 other dogs	130	174	+42	111	115		1.87

* Na taurocholate.

30 per cent; the optimum was 65 per cent (fig. 3). It is interesting that this figure for optimum recovery is the figure for optimum recovery of dog's bilirubin introduced into the intestine in the experiments of McMaster and Elman (13).

McMaster and Elman (13) and Broun, McMaster and Rous (14) observed an increase in the elimination of bile pigment in the bile after feeding bile orally. They interpreted their evidence as indicating an enterohepatic circulation of bilirubin. The former investigators realized that the increased output of pigment might be due to the choleretic effect

of the bile salts in the bile; so they gave four dogs from 2 to 5 grams of sodium glycocholate orally. Neither they nor Wisner and Whipple (15) observed an increase in bile pigment output.

We have studied the effect of cholic acid in the form of ox-bile salts and also sodium taurocholate given orally with a meal twice daily on the output of bile pigment in dogs prepared by the Rous-McMaster method. Although daily outputs for successive six or seven-day periods may be

TABLE 4

Showing the effect of atropine and beta methyl acetylcholine on bile output

After obtaining a 2 hour ante cibum control, the dogs were fed a meal without the return of bile. The dogs were on "suction." The results in the three dogs used are averaged.

HOURS	VOLUME	CHOLIC ACID	CHOLESTEROL	PIGMENT
Control				
		mgm.	*mgm.*	*mgm.*
2 hr. A. C.*	18.1	142	1.01	8.2
1st and 2nd hr. p. c.	20.7	200	1.11	12.7
3rd and 4th hr. p. c.	29.1	228	1.32	15.2
5th and 6th hr. p. c.	19.8	156	0.80	7.8
Atropine 1 mgm. subcutaneously 30 minutes p. c.				
2 hr. A. C.	18.0	140	1.01	6.0
1st and 2nd hr. p. c.	18.5	172	1.02	9.8
3rd and 4th hr. p. c.	18.9	184	1.11	10.5
5th and 6th hr. p. c.	19.8	166	1.16	10.4
Mecholyl 0.3 mgm. every 20 minutes for 3 hours subcut.				
2 hr. A. C.	18.2	162	1.12	10.0
1st and 2nd hr. p. c.	19.1	174	1.19	11.6
3rd and 4th hr. p. c.	19.5	196	1.36	12.2
5th and 6th hr. p. c.	21.9	194	1.32	12.7

* "A. C.", ante cibum.

quite constant, the fluctuation from one period to another separated by a month or more may be ± 35 per cent of the mean by their method in our hands. Typical data are given in table 3, and illustrate the experimental variations and at the same time confirm the observations of the investigators cited above (13, 14, 15). It should be pointed out that these data do not mean that bile salts will not increase the elimination of bile pigment when bilirubin is present in *excessive* quantities in the blood.

Bollman, Sheard and Mann (16) using dogs under ether or local anesthesia and analyzing portal and systemic blood for bilirubin spectrophotometrically, could find no increase in bilirubin in the portal blood after the introduction of bile into the intestine. Blankenhorn (17) using an angiostomy technique on unanesthetized dogs, was unable to demonstrate the absorption of bilirubin into the portal blood, although his evidence indicates that bilirubin might be absorbed to a very slight extent by the lymphatics. Urobilin was absorbed from the intestine into both the blood and lymph. Sockey, Johnston, and Ravdin (18) also failed to observe the absorption of bilirubin from a jejunal fistula.

Since the methods used by Bollman and by Blankenhorn were sufficiently sensitive to detect the small amount of bilirubin *apparently* absorbed from the intestine in the experiments of McMaster and Elman (13) and of Roger (19), the extra bile pigment eliminated in our experiments when bile was returned *a*, must have been absorbed into the lymph, or *b*, must represent urobilin that has been absorbed and converted to bile pigment by the liver, or *c*, the bile pigment in bile is some special form of bilirubin other than the "indirect reacting" pure bilirubin usually used for absorption studies.

Atropine and beta methyl acetylcholine. Three dogs were studied by the suction method. They were placed on a 7-7-10 hour feeding schedule without the return of bile. The bile was collected for the last 2 hours during the ten-hour period. Then the usual ration was fed without the administration of the drug. The next day the same regimen was repeated but the drug was given. One milligram of atropine was injected subcutaneously one-half hour after feeding and 0.3 mgm. of beta methyl acetylcholine (mecholyl) was given every 20 minutes for 3 hours after the meal.

The results were so similar on each dog that they have been averaged and presented in table 4. It is to be noted that atropine abolished the characteristic secretory response (fig. 2); recovery from the effects of the drug are apparent during the 5th and 6th hour period. An increase in cholic acid output occurred after each drug, but the increase was less than the control response, showing that cholic acid synthesis was also depressed by both drugs. Of course, it cannot be stated that the observed effects of the drugs are due to their direct action of the liver.

In view of the observations of Tanturi and Ivy (20) on the secretory nerves of the liver, it was thought that atropine and beta methyl acetylcholine might exert a greater effect than that observed on the rate of secretion of bile.

SUMMARY AND CONCLUSIONS

The effect of various procedures on the composition of bile has been studied in biliary fistula dogs.

1. Under standard conditions of feeding with or without the return of bile to the duodenum, the volume and cholic acid output did not deviate more than ± 8 per cent from the mean daily values; the cholesterol and total pigment output was subject to ± 20 per cent deviation. When the Rous-McMaster method is used, which does not employ suction to overcome the resistance to the flow of bile caused by the drainage tubes, etc., the volume output is less and is subject to greater variation. The same is true of cholic acid output, but significant results may be obtained by their method in so far as the output of the various principal biliary constituents are concerned.

2. When an animal is fed three times daily without the return of bile, the output of cholates, cholesterol and pigment is quite constant for three periods. When bile is returned after each meal, the percentage increase in volume, cholesterol and pigment output is essentially the same, but the output of cholates is markedly increased.

3. The cholic acid secreted daily by the dogs (8 to 15 kilos in weight) when receiving the diet alone ranged from 1.2 to 1.6 grams; when all the bile was returned to the duodenum from 6.5 to 9.0 grams were secreted. From 10 to 15 per cent of the cholic acid disappears during the enterohepatic circuit. Thus, if from 7 to 9 grams of cholic acid traversed the enterohepatic circuit three times each day, the total loss would compensate for that formed endogenously and from the diet, and a more or less constant output of cholic acid would be maintained.

4. When bile was returned, the output of cholesterol was increased and from 33 to 66 per cent of the cholesterol introduced in the bile appeared to be absorbed and excreted. Bile salts, containing no cholesterol administered orally or intravenously increased cholesterol output. Thus a relation between volume and cholesterol output exists, although the relation is not a strict one.

5. When bile salts, containing no pigment, were given orally the output of pigment was not significantly increased. However when bile was returned to the intestine, it appeared that about 30 per cent of the pigment was in some way absorbed and re-excreted by the liver.

6. The subcutaneous administration of 1 mgm. of atropine and of 2.7 mgm. of beta methyl acetylcholine, the latter being given in divided doses every 20 minutes for 3 hours, prevented the usual increase in volume and the cholic acid content of the bile that occurs after a meal.

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THE EFFECT OF PARATHYROID HORMONE ON THE PERMEABILITY OF THE LENS CAPSULE TO CALCIUM

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In a recent publication (1) it was found that lens protein, extracted in 0.5 per cent KCl at pH 7.2, showed no increase in opalescence after exposure to ultraviolet radiation at 4°C., followed by heating to a moderate temperature (40°C. for 40 minutes). However, extracts in 0.5 per cent KCl or Ringer solution, containing varying amounts of $CaCl_2$, developed an opalescence when radiated and heated which increased with the amount of calcium present.

These results suggested that exposure of the eyes to ultraviolet radiation (λ 300–315 mμ), which penetrates the cornea but is absorbed by the lens, would be capable of denaturing the lens protein so that opalescence would develop provided small amounts of calcium salts were present in the lens. The problem of the etiology of cataract was therefore continued by studying the permeability of the lens capsule to calcium under conditions which might produce a change in the normal permeability.

Friedenwald (2) found that the permeability of the isolated lens capsule decreased with age and with increasing molecular weight of the diffusing substance. He suggested that senile cataract is associated with a decrease in capsular permeability while other observers have assumed that cataract formation may be related to an increase in permeability. In contrast to the isolated capsule the living lens is probably not permeable to all substances of low molecular weight. The low concentration of calcium in the normal lens is evidence of the fact that the capsule is normally impermeable to calcium and any condition which would tend to make the lens more permeable to calcium would undoubtedly favor the formation of cataract.

METHOD. Pig lenses were removed without injuring the capsule and immersed for one hour in Ringer solution containing 0.1 per cent $CaCl_2$. In some experiments Ringer containing 0.25 per cent $CaCl_2$ was used and substances were added to these solutions which might be expected to affect the permeability of the lens capsule to calcium. After having been immersed for one hour the lenses were removed, washed in 0.5 per cent KCl, and extracted in 0.5 per cent KCl (7 cc. per lens). The extracts (pH 7.2)

were exposed to the total radiation of a quartz mercury arc in shallow open dishes on ice, at a distance of six inches for 35 minutes and were kept at a temperature of 4°C. during the radiation. There was no appreciable increase in opalescence due to radiation alone, or to moderate heating alone, but when the radiated extracts were heated to 40°C. for 40 minutes opalescence developed which was proportional to the amount of calcium in the lens. Comparison of the opalescence with that obtained in lens extracts containing known amounts of $CaCl_2$ (fig. 1) gave a method of determining the amount of calcium that had penetrated the lens capsule.

The opalescence of the solutions was measured by means of a Tyndallmeter described in detail in a previous publication (3). The intensity of the Tyndall beam in apparent foot-candles, as read by a Macbeth Illumin-

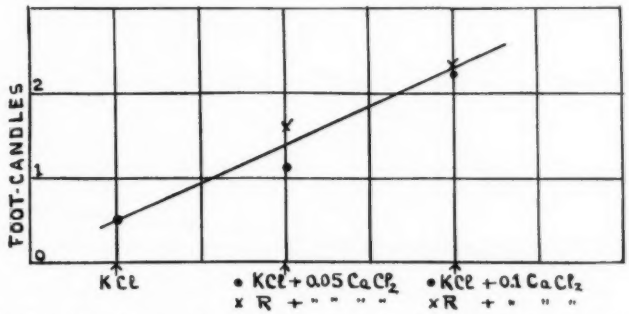

Fig. 1. Opacity of lens extracts exposed to ultraviolet radiation and heated to 40°C. for 40 minutes.

● Extracts in 0.5 per cent KCl and in 0.5 per cent KCl with 0.05 and 0.1 per cent $CaCl_2$ added.

× Extracts in Ringer containing 0.05 and 0.1 per cent $CaCl_2$.

Ordinates: intensity of Tyndall beam in apparent foot-candles.

ometer, gives a quantitative measure of the concentration of aggregated protein when the solutions are near the isoelectric point. At the hydrogen ion concentration of the lens the readings cannot be interpreted in grams of aggregated protein although they may be taken as a measure of the opalescence of the solutions.

All experiments were carried out at room temperature.

Results. Almost negligible amounts of calcium penetrated the capsule of lenses from freshly enucleated eyes during one hour's immersion in Ringer containing 0.1 per cent $CaCl_2$. If the eyes were kept in the icebox a day or longer the permeability to calcium, as measured by the increasing opalescence after radiation, increased at a definite rate. These results gave a control curve which is shown in figure 2. In studying the effect of substances which might be expected to increase permeability to calcium,

lenses from fresh eyes were used and the results compared with the normal value of the Tyndall beam of 0.65 foot-candles. In studying the effect of substances which might decrease the permeability to calcium, lenses from day old eyes were used which gave an average normal Tyndall beam reading of 0.99 foot-candles. The control curve was the same if Ringer plus 0.25 per cent $CaCl_2$ was used.

Ultraviolet radiation. It has frequently been stated that exposure to ultraviolet radiation increases the permeability of cell membranes. Lenses from fresh eyes were exposed to ultraviolet radiation from the quartz

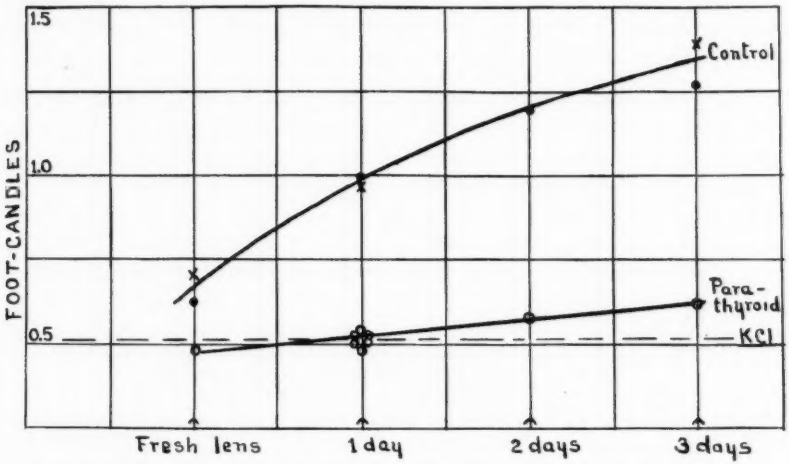

Fig. 2. Opacity of lens extracts radiated and heated to 40°C. for 40 minutes. — — Extract in KCl. Control curve = extracts of lenses after immersion in ● Ringer plus 0.1 per cent $CaCl_2$, × Ringer plus 0.25 per cent $CaCl_2$.

○ Extracts of lenses after immersion in Ringer plus 0.1 per cent $CaCl_2$ and 5 units of parathyroid extract per 8 cc.

Abscissae: age of eye after enucleation.

Ordinates: intensity of Tyndall beam in apparent foot-candles.

mercury arc during the hour's immersion in Ringer plus 0.1 per cent $CaCl_2$. There was no change in permeability to calcium.

Hydrogen ion concentration. Slight changes in acidity (pH 6.0–8.0) had no effect on the permeability of the lens to calcium.

Viosterol. From one to three drops of viosterol added to 10 cc. of the Ringer plus 0.1 per cent $CaCl_2$ solution, in which the lenses were immersed, had no effect on the permeability of the lens capsule to calcium.

Ascorbic acid. Ascorbic acid was added to the Ringer plus 0.1 per cent $CaCl_2$ solution in amounts varying from 20 units to 80 units per 8 cc. and the solutions adjusted to pH 7.2. The lenses from fresh eyes showed no increase in permeability to calcium in these solutions.

Parathyroid extract. Parathyroid extract (Lilly) was added to the Ringer plus 0.1 per cent $CaCl_2$ and produced a marked decrease in the permeability of the lens capsule to calcium. Figure 2 shows that addition of 1 drop (5 units) of parathyroid extract to 8 cc. of solution completely prevented the penetration of calcium into the lens both in fresh eyes and in those kept from one to three days in the ice-box. These results were repeated with five samples of the extract in two successive years and with the exception of one sample, which had apparently lost its potency and gave irregular results, the experiments all gave identical results and the readings shown in figure 1 represent averages from more than a hundred determinations.

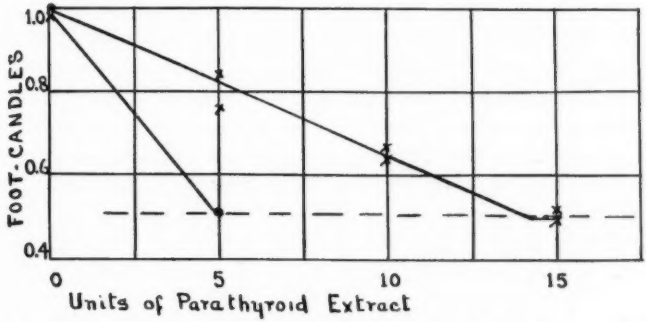

Fig. 3. Opacity of extracts of lenses after immersion in ● Ringer with 0.1 per cent $CaCl_2$, × Ringer with 0.25 per cent $CaCl_2$ to which varying amounts of parathyroid extract have been added. Extracts radiated and heated to 40°C. for 40 minutes.

— — Opacity of extract in KCl containing no calcium.

Abscissae: units of parathyroid extract per 8 cc. of solution.

Ordinates: intensity of Tyndall beam in apparent foot-candles.

If lenses were immersed in Ringer solution containing 0.25 per cent $CaCl_2$ there was no difference in the control curve but it took more parathyroid extract to completely prevent the penetration of calcium into the lens as is shown in figure 3. While one drop (5 units) per 8 cc. completely prevented the entrance of calcium in a concentration of 0.1 per cent it took 13 or 14 units per 8 cc. to prevent the penetration of calcium in a concentration of 0.25 per cent. This would indicate that the parathyroid extract acts directly on the calcium by binding it in some non-diffusible form and indeed a slight opalescence develops when parathyroid extract is added to a solution containing calcium chloride, the degree of opalescence being proportional to the amount of parathyroid extract. However, as the effect of 0.1 per cent and 0.25 per cent $CaCl_2$ was the same in the control curve one would expect that addition of one drop of parathyroid ex-

tract to the solution containing 0.25 per cent $CaCl_2$ would still leave at least 0.1 per cent $CaCl_2$ uncombined and consequently would have no measurable effect. The results therefore cannot be clearly related to the formation of a non-diffusible calcium-hormone complex although that seems the most reasonable assumption.

Discussion. The results given here indicate that the presence of parathyroid hormone in the body fluids prevents the penetration of calcium into cells. The fall in serum calcium in parathyroid deficiency, or after parathyroidectomy, may therefore be due to the passage of calcium from the serum to cells. This would explain the experimental fact that after parathyroidectomy there is no increase in calcium excretion and that the bodies of the animals contain more calcium than the controls. Some observers have reported an increased calcium content of the muscles, some of the red blood cells, although these results have not been confirmed by other investigators. A greater penetration of calcium into cells in parathyroid deficiency would account for the occurrence of cataract which usually follows parathyroidectomy. Bourne (4) states that although cataract occurs after parathyroidectomy it does not accompany hypocalcemia occurring in other diseases, which bears out the idea. Shelling (5) states that he never saw a cataract in over 200 operated rats but adds that these animals were kept out of direct sunlight. This substantiates the theory (1) that one would only expect calcium in the lens to produce a cataract if the lens protein has been denatured by radiation.

Some interesting studies on parathyroprival cataract in rabbits were made recently at the University of Naples (6, 7). In the animals with cataract there was a reduction in oxygen consumption and glycolysis of the lens, no change in the ascorbic acid or glutathione content of the lens, a decrease in calcium in the aqueous humor and a marked increase of calcium in the lens. The authors conclude that changes in the capsular epithelium make the lens more permeable to calcium and favor precipitation of the lens proteins, but in view of the results presented in this paper the increase in calcium in the lens might be attributed directly to the deficiency of parathyroid hormone in the blood and aqueous humor.

Cameron and Moorhouse (8, 9) originally suggested that there was a non-diffusible calcium-hormone complex in the blood but later gave up this idea because it was found that there is little change in the level of cerebro-spinal fluid calcium after parathyroidectomy. However, as the capillary walls are normally more permeable than cell membranes one may assume a calcium-hormone complex in the blood which diffuses more or less readily through capillary walls but not through the lens capsule or the red cell membrane.

The first effect of the injection of parathyroid extract is the excretion of phosphorus in the urine. There would seem therefore to be a balance

between a calcium-hormone complex and a calcium-phosphate complex in the blood, the first diffusible through capillaries but not through cell membranes and the second in equilibrium with the calcium phosphate in bone. A decrease in hormone would free calcium from the calcium-hormone complex and favor increased penetration of calcium into cells, especially if the animal is on a high calcium diet, and also increase the amount of calcium phosphate if on a high phosphorus diet, thereby favoring bone formation and bringing about a decrease in total serum calcium. An increase in hormone would increase the amount of the calcium-hormone complex, remove calcium from the calcium-phosphate complex, lead to increased excretion of phosphorus and bring calcium phosphate from the bones into the serum, thereby raising the level of total serum calcium. It is known that parathyroid hormone is an unfavorable treatment for rickets and inhibits calcification in experimental fractures. According to this suggestion a very delicate balance of parathyroid hormone is necessary, an excess favoring rickets in infancy and a deficiency favoring cataract at any age.

CONCLUSION

Pig lenses were immersed in solutions containing calcium and the rate of penetration of calcium into the lens was determined by the degree of opalescence in the lens proteins when extracted in KCl and exposed to ultraviolet radiation and moderate heat. When parathyroid extract was added to the solutions in which the lenses were immersed the penetration of calcium into the lens was completely prevented. This suggests that the low blood calcium after parathyroidectomy is due to loss of calcium to the cells and that the cataracts occurring after parathyroidectomy are due to a combination of two causes: 1, denatured lens protein if the eyes have been exposed to ultraviolet radiation, and 2, greater permeability of the lens capsule to calcium in the absence of parathyroid hormone.

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HEMOGLOBIN PRODUCTION FACTORS IN THE LIVER

Fish, Frog and Turtle Compared with Domestic Animals

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When we observed (1) that fish liver appeared to contain very little of the material out of which the standard anemic dog can produce new hemoglobin, we suspected that the lower forms of vertebrates might be alike in this respect. Opportunity to test this suspicion did not come until recently. Meanwhile occasional tests of mixed fish liver material from fish markets gave irregular values (table 3) and it was decided to test carefully fresh shark liver (table 1). Shark liver is deficient in hemoglobin production factors and is but little more than half as potent as the standard pig liver. It is apparent that fresh *turtle* and *frog liver* have a substantial content of hemoglobin production factors—that in this respect they are close to beef liver which ranks lowest among the domestic animal group carefully tested (2).

We are not certain why our early experiments with mixed fish livers gave such low figures for hemoglobin production but suspect that the fish livers autolyzed (due to inadequate or delayed refrigeration) with loss of the materials which effect hemoglobin production in the standard anemic dog.

We may refer (2) to careful standardization of the livers of common domestic animals. If we take the pig liver as our normal base line and rate it as 100 per cent for new hemoglobin production, then the beef liver is low (70 per cent) and the horse liver is high (130 per cent). In like manner rabbit liver rates 80 per cent and reindeer liver averages 90 per cent. Dog liver is equivalent in potency to pig liver.

We may recall (3) that the human liver when normal may contain abundant material out of which new hemoglobin can be produced by the standard anemic dog. In fact compared for potency with pig liver (100 per cent) the normal human liver rates 160 per cent. Even in long continued secondary anemia (4) the human liver does not part with this material and rates 135 per cent, although the iron stores are depleted. In pernicious or aplastic anemia the hemoglobin building material heaps up in the liver because there is no outlet for its utilization and we observe values of over 200 per cent.

TABLE 1

Shark liver contains about two-thirds the potency of pig liver

DIET PERIODS 1 WEEK EACH FOOD, GRAMS PER DAY	FOOD CONSUMED	WEIGHT	PLASMA VOL.	BLOOD Hb. LEVEL	BLOOD Hb. REMOVED	NET Hb. PRODUCTION
Dog 29-326. Coach, male, adult						
	per cent	*kgm.*	*cc.*	*per cent*	*grams*	*grams*
Bread 300, salmon 50, Klim 20	100	17.4	951	43	1.2	
Liver 175, bread 250, Klim 20	81	17.5	997	42	20.7	
Liver 160, bread 250, Klim 20	95	17.8	1000	39	1.0	
Bread 300, salmon 50, Klim 20	100	17.8	890	42	1.2	(59)
Bread 300, salmon 50, Klim 20	100	17.9	930	47	10.5	29.0
Dog 35-4. Bull, male, adult						
Bread 500, salmon 50, Klim 20	100	18.5	1136	43	1.2	
Liver 220, bread 350, Klim 20	100	18.4	1080	46	1.3	
Liver 220, bread 350, Klim 20	100	18.6	1107	52	25.6	
Bread 500, salmon 50, Klim 20	100	18.7	1066	53	13.1	(45)
Bread 500, salmon 50, Klim 20	100	18.4	1067	43	1.2	33.0
Dog 32-5. Coach, female, adult						
Bread 350, salmon 50, Klim 20	100	15.8	927	48	1.4	
Liver 270, bread 250, Klim 20	86	15.8		42	25.4	
Liver 216, bread 250, Klim 20	66	15.1	728	42	13.0	
Liver 171, bread 250, Klim 20	93	15.5	754	38	31.7	
Bread 350, salmon 50, Klim 20	100	16.0	938	40	1.1	(57)
Bread 350, salmon 50, Klim 20	100	16.2	979	40	1.1	57.0
Dog 33-14. Coach, female, adult						
Bread 250, salmon 100, Klim 20	100	12.3	791	32	0.9	
Liver 186, bread 250, Klim 20*	76	12.2	675	49	1.3	
Liver 200, bread 250, Klim 20*	92	12.6	700	45	19.6	
Bread 300, salmon 50, Klim 20	100	12.7	767	46	1.3	(64)
Bread 300, salmon 50, Klim 20	100	12.6	717	38	14.0	35.0

Net hemoglobin production figures in ( ) indicate output as calculated for a *two weeks feeding of 300 grams liver* per day.

* Fed uncooked.

METHODS. All method details relating to the general anemia program have been described recently (5) and need not be repeated. *Net hemo-*

globin production means the output of new hemoglobin which is attributable to the supplemental food factor and it is calculated as follows. The gross hemoglobin output for any given period includes all blood withdrawn, even the small samples taken for hematocrit estimations. From this gross total we subtract the hemoglobin which is due to the basal bread ration as determined by long periods on the basal diet alone. Finally we make an adjustment for the difference in hemoglobin level in the dog at the start and at the end of each experimental period—usually a five week interval of which 2 weeks cover the feeding period and 3 weeks include the after period during which any "carry over" is exhausted. A ten kilo dog will have in its circulation about 55 grams hemoglobin at an anemia level of 50 per cent. We therefore allow 1 gram of hemoglobin for each per cent difference in hemoglobin level and know by experience that this is a close approximation. Let us say that a dog shows a 50 per cent hemoglobin level at the start of the experimental period and during the after period it is bled a little too much giving a 44 per cent hemoglobin level at the end of the experimental period. Obviously this 6 grams hemoglobin is not due to hemoglobin production but to over-bleeding and is therefore deducted from the gross total hemoglobin to give the net hemoglobin production.

All animals used in these experiments were in a normal state of health. Hemoglobin values of 100 per cent = 13.8 grams hemoglobin per 100 cc. of blood.

Fish liver because of its high oil content is not relished by dogs and many animals will not eat it whether cooked or raw. In some experiments the dog ate fish liver for a few days and then refused. These incomplete experiments are not recorded. Often some of the liver was refused. The food consumption is listed in table 1. When correction is made for lessened intake it may give figures a little too large since the dog as a rule utilizes smaller amounts of food to slightly better advantage than when large amounts are fed daily.

Fish liver as fed in table 3 was a mixture of codfish, haddock, hake and pollack livers. This material was obtained from a Boston fish dealer and when received was soft and very oily. It was difficult to say whether the livers were well preserved or not. We cannot say as to the promptness of shipment after removal from the market fish.

Shark livers were obtained from Shark Industries, Florida, which markets vitamin products made from shark liver. These shark livers arrived in excellent condition after prompt shipment by express refrigerated with ice. The livers were obtained from the common sharks of that vicinity—listed by the firm as "leopard, mackeral, lemon, hammerhead, sand, dusky, nurse and tiger."

Turtle livers were obtained from "Loggerhead" turtles from fresh

water—live weight 20 to 80 lbs; individual livers, fresh weight, 150 to 310 grams. Fresh weight is given in table 2. Iron content of fresh liver = 15 mgm. per cent. The material was shipped promptly from Rayne, Louisiana, by express, refrigerated with ice, and reached us in excellent condition. Turtle and frog livers are not oily and are eaten with relish.

Frog livers were obtained from large "bull frogs" used for market sale of frog legs. Live weight of frog 2 to 3 lbs. Individual weight of fresh liver 12 to 21 grams. Iron content of fresh liver = 13 mgm. per cent. The material was shipped promptly from Rayne, Louisiana, by express, refrigerated with ice, and reached us in excellent condition.

TABLE 2

Frog and turtle livers approximate the potency of beef liver

DOG NO.	LIVER ADDED TO BASAL BREAD RATION		CONTROL NET HEMOGLOBIN OUTPUT, GRAMS PER 2 WKS.		
	Food, grams per day	Hemoglobin net output per 2 wks.	Iron 40 mgm. daily—oral	Pig liver 300 grams daily—oral	Basal bread ration alone
			Frog liver		
		grams			
35-6	Liver 300, bread 350, Klim 20	92	57	107	30
30-116	Liver 300, bread 300, Klim 20	50	42	92	20
35-4	Liver 300, bread 350, Klim 20	80	56	89	4
			Turtle liver		
35-7	Liver 300, bread 200, Klim 20	73	49	89	4
33-13	Liver 300, bread 250	101	43	103	36
29-67	Liver 300, bread 250	69	66	101	18

EXPERIMENTAL OBSERVATIONS. Table 1 shows four satisfactory experiments with *shark liver* added to the basal ration. The amounts of liver given depend upon the liking of the dog for the liver. In the third dog (32-5) the liver was given during 3 weeks in an amount equal to 300 grams daily intake for 2 weeks. The total net production of hemoglobin was 57 grams which contrasts with 91 grams hemoglobin which is the control figure for pig liver in the same dog. If we correct for the smaller liver intake and figure the hemoglobin production on the basis of 300 grams intake per day for 2 weeks, we find an average output for the four experiments of 56 grams hemoglobin to compare with average production by the same dogs on pig liver (300 grams daily for 2 weeks) of 84 grams hemoglobin.

In all these experiments (table 1) the standard salmon bread noted in the tables contained 3.2 mgm. iron per 100 grams bread and contained no bran. This accounts for the consistently low basal output per week on the basal ration—2 grams hemoglobin per week.

Table 2 gives three satisfactory experiments with frog liver. All food was promptly eaten and the production of new hemoglobin in the standard anemic dogs is considerable—in fact is equivalent to the production observed after feeding equal amounts of beef liver. These three dogs show an average production of 74 grams hemoglobin due to frog liver and the pig liver control gives an average production of 96 grams in the same dogs. The general reaction to the frog and pig liver is very similar. The iron content of the fresh frog liver is 13 mgm. per cent as compared with 17 mgm. per cent for pig liver.

TABLE 3

Mixed fish livers show wide variations in potency

DOG NO.	FISH LIVER ADDED TO BASAL BREAD RATION		CONTROL NET HEMOGLOBIN OUTPUT, GRAMS PER 2 WKS.		
	Food, grams per day	Hemoglobin net output per 2 wks.	Iron 40 mgm. daily—oral	Pig liver 300 grams daily—oral	Basal bread ration alone
		grams			
33-13	Liver 300, bread 250, Klim 20	18	43	103	30
30-121	Liver 300, bread 350, Klim 20	31	54	98	28
30-117	Liver 300, bread 300, Klim 20	32	57	77	46
32-5	Liver 300, bread 250, Klim 20	45	69	86	36
33-14	Liver 300, bread 300, Klim 20	72*	58	66	20

* 1 week feeding only—net hemoglobin output corrected for 2 weeks.

Table 2 also shows three satisfactory experiments with turtle liver which reacts like frog or beef liver but has a slightly higher content of hemoglobin producing factors. The turtle livers were eaten promptly and the average output resulting from 2 weeks' feeding of 300 grams liver daily is 81 grams hemoglobin as compared to the control values for pig liver feeding of 98 grams hemoglobin in the same dogs. The iron content of the fresh turtle liver is 15 mgm. per cent.

It may be argued that the frog and turtle liver or pig liver are not as superior to fish liver as these feeding experiments indicate. The fish liver contains a very large amount of fat which "dilutes" the protein fraction in the fish liver. The potency of liver for hemoglobin regeneration in anemia due to blood loss is believed to reside largely in the protein fraction and is precipitated by alcohol (6)—not present in the fat.

The protein content of mixed market fish liver (table 3) is about 7.3 per cent, of frog liver about 22 per cent, and of pig liver about 21 per cent.

Obviously if we correct for this "dilution" of the liver protein largely due to fat, the fish liver protein compares favorably with beef liver and pig liver protein as regards its content of hemoglobin building materials.

Table 3 gives some of the more recent experiments in which mixed fish liver (see Methods) was fed to anemic dogs. Some earlier experiments (1) indicated that this fish liver contained very little of the material which contributes to hemoglobin building in the standard anemic dog. We note in table 3 great variations in the amount of new hemoglobin produced as a result of fish liver feeding. It is possible that postmortem autolysis may have been a factor but it is also probable that the fat content may have been a factor as the potent material is not present in the fat which actually "dilutes" the protein and its contained potent material for new hemoglobin construction.

It will be noted that the basal bread ration in table 3 gave very high values as compared with table 1. The reason for this was that this particular salmon bread contained bran and much more iron—9 mgm. per cent. All fish liver was consumed in these experiments with a single exception of dog 32-5 which consumed 94 per cent of the liver and the hemoglobin output is corrected for 100 per cent consumption.

SUMMARY

Reptilian liver (turtle) contains a liberal amount of hemoglobin producing factors as tested in the standard anemic dog. This liver contains a little more of these potent hemoglobin building factors than does beef liver.

Amphibian liver (bull frog) contains a little less of these potent hemoglobin building factors and rates about on a par with beef liver.

Fish liver rates still lower but shows wide variations in the content of hemoglobin building factors. These variations may be due in part to postmortem digestion or to high fat content which "dilutes" the protein contained hemoglobin building factors.

Shark liver is more uniform in these tests as compared with the liver derived from various market fish but it shows a content of hemoglobin building factors somewhat below that of beef liver. The earlier experiments with fish liver which indicated very low potency are probably to be explained in large part by postmortem autolysis.

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HYPOPHYSEAL AND ADRENAL INTERRELATIONSHIPS AND CARBOHYDRATE METABOLISM

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Evidence from many sources has indicated that carbohydrate losses following hypophysectomy may not be due to absence of pituitary factors alone, but that adrenal hypofunction may be in part responsible for such carbohydrate depletion. We have been concerned with this phase of hypophyso-adrenal synergy in experiments conducted during the last three years in this laboratory. Reports of these investigations have already been made in abstract (Corey, 1937, 1938). The present paper deals with the results of earlier studies together with those of more recent date.

In order to evaluate the rôle of the adrenal cortex in maintaining carbohydrate levels in the body, hypophysectomized male rats were fasted for different time periods, during which they were injected intraperitoneally with highly purified cortico-adrenal extracts made in this laboratory (Ehrenstein and Britton, 1937).

About 100 hypophysectomized rats fasted for 48 hours were injected with extract in varying amounts. These animals exhibited blood sugar levels equal to, or in excess of (av. 90 mgm. per cent), those seen in unoperated controls. Bioassay of our extracts by the blood-pressure method failed to reveal the presence of adrenalin or choline. Also blood sugar curves obtained by hourly sampling, following extract or adrenalin injection, gave evidence that the augmented sugar levels observed in the extract-treated animals were not attributable to adrenalin, since the response to extract injection was greater and of much longer duration, as shown in figure 1.

In these early experiments the glycogen content of the liver showed no significant increase under the influence of the extract, and muscle glycogen concentration remained unaffected, whether or not the hypophysectomized animals were treated with extract or adrenalin (or even glucose).

As one of us stated at this time (Corey, 1938), the invariably high blood sugar levels observed in the extract treated animals of this extended series were regarded as highly significant. They were apparent even in rats

receiving as little as 3 cc. of extract twice daily, and higher dosages up to 5 cc. thrice daily produced no greater elevations of blood sugar, although tissue glycogen values remained unaffected.

The possibility of a refractory condition of the organism which might become relatively chronic, as regards tissue glycogen formation during the comparatively long (48-hour) fasting period employed, appeared probable. A series of experiments was thus carried out in which the fasting period was shortened to 24 hours, injections of extract being made during this time in increasing amount or with greater frequency. The results of these experiments are summarized in table 1.

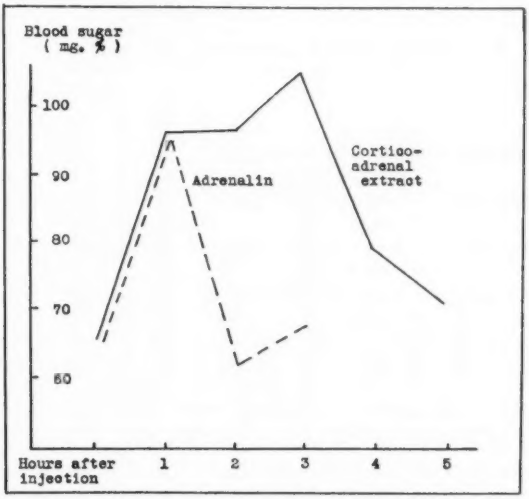

Fig. 1. Showing the effect on the blood sugar of hypophysectomized rats of injections of 6 cc. of cortico-adrenal extract or 1:100,000 adrenalin chloride. Four animals were employed in obtaining each curve.

It is interesting to note that in the case of those animals which received the least amount of extract at the longest time intervals (3 cc. twice daily), the blood sugar levels averaged considerably above those found in the normal, unoperated controls—i.e., 93 mgm. compared to 74 mgm. per cent. Liver and muscle glycogen values nevertheless were unaffected in these cases. Larger injections of extract administered more frequently (5 cc. three times daily) produced no more significant changes in blood sugar, and only slight elevations of liver and muscle glycogen levels. No quantitative correlation was therefore apparent under these conditions between the amount of extract administered and the carbohydrate response. The same amount of extract (5 cc.) injected five times daily gave the first

significant evidence of tissue glycogen storage. Hourly injections of small quantities of extract (1 cc.) produced glycogen synthesis in the liver considerably in excess of that seen in unoperated controls. It may be observed that muscle glycogen concentration was not wholly restored to

TABLE 1

Effects of cortico-adrenal extract on carbohydrate levels in hypophysectomized rats

All operations were carried out from 7 to 9 days previous to a 24-hour period of fasting, during which treatment was administered.

NO. OF RATS	EXPERIMENTAL CONDITIONS	TREATMENT	TOTAL AMOUNT OF EXTRACT INJECTED	BLOOD SUGAR	LIVER GLYCOGEN	MUSCLE GLYCOGEN
		A. Injections started at beginning of fasting period				
			cc.	*mgm. per cent*	*grams per cent*	*grams per cent*
12	Normal	None		74	0.46	0.31
12	Hypophysectomized	1 cc. 0.9 per cent NaCl every hour		60	0.14	0.23
6	Hypophysectomized	3 cc. extract* twice daily	6	93	0.12	0.26
11	Hypophysectomized	5 cc. extract three times daily	15	95	0.15	0.27
10	Hypophysectomized	5 cc. extract five times daily	25	109	0.26	0.27
12	Hypophysectomized	1 cc. extract every hour	24	93	0.72	0.25
		B. "Replacement" experiments. All animals fasted for 12 hours, after which extract injection was begun and continued for 12 hours. Other conditions as above				
4	Hypophysectomized	None. Fasted 12 hours		69	0.22	0.28
2	Hypophysectomized	4 cc. 0.9 per cent NaCl every 2 hours		60	0.16	0.24
5	Hypophysectomized	4 cc. extract every 2 hours	24	99	0.31	0.23
2	Hypophysectomized	5 cc. extract every 2 hours	30	91	0.52	0.28

* "Extract" refers to cortico-adrenal extract prepared in this laboratory, in which each cubic centimeter represented 40 grams of fresh beef adrenal.

normal by the extract, although there was definite evidence of some degree of restoration.

After ascertaining that both blood sugar and liver glycogen levels in hypophysectomized rats might be raised above those seen in normal controls by means of cortico-adrenal extract administration, "replacement" experiments were carried out in which hypophysectomized animals were

first fasted for 12 hours; cortico-adrenal extract injection was then begun and continued for 12 hours. The animals were thereafter utilized as above. To ensure that adequate amounts of extract be administered, injections of 4 cc. or 5 cc. were given at 2-hour intervals (table 1, B). In all cases the extract proved effective, both as regards blood sugar and liver glycogen changes, and with the larger dosage liver glycogen levels exceeding those seen in the saline-injected controls were demonstrated.

It was thus apparent that the lowered blood sugar and liver glycogen concentrations which follow fasting in the hypophysectomized rat may be elevated to normal (or supernormal) levels by means of injections of active cortico-adrenal extracts, administered in adequate amount and at sufficiently frequent intervals.

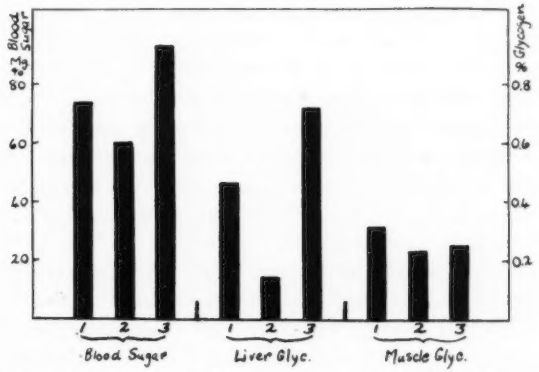

Fig. 2. Carbohydrate levels in 24-hour fasted rats under different conditions: 1, normal controls; 2, hypophysectomized, untreated; 3, hypophysectomized and treated with cortico-adrenal extract (see text).

Discussion. That both the hypophysis (Houssay, *et al.*, 1931, 1932; Russel, 1936; Chaikoff, *et al.*, 1935; Phillips and Robb, 1934; Smith *et al.*, 1936; Fisher and Pencharz, 1936; and others) and the adrenal cortex (Britton, 1932; Britton and Silvette, 1934, 1937; Long, 1935; Hartman and Brownell, 1934; Corey and Britton, 1937) are intimately concerned with carbohydrate economy has now been definitely established. Similar results which followed either hypophysectomy or adrenalectomy (Long and Lukens, 1936) have led to the suggestion (Long) that the hypophysis may control sugar formation from protein through the mediation of the adrenal glands.

Fully-fed hypophysectomized rats show no carbohydrate depletion, but under the same experimental conditions subsequent adrenalectomy brings about markedly decreased sugar and liver glycogen levels. This led us

(Corey and Britton, 1937) to express the view that cortico-adrenal atrophy (Smith, 1930; Cutuly, 1936) might be a major factor in bringing about carbohydrate reductions in fasted, hypophysectomized rats. Bennett (1937) has reported hyperglycemia and increased glycogen values in hypophysectomized rats following the prolonged administration of the adrenocorticotropic hormone of the pituitary. These considerations made it appear possible that the administration of cortico-adrenal extracts to fasted, hypophysectomized rats might prevent, partially or completely, the occurrence of carbohydrate deficiencies. It was with this idea that we first took up this work in 1936 (reported, 1937, 1938, in abstract).

It is apparent from the results that injections of extract must be made in considerable quantities and at short, hourly or two-hourly intervals, if tissue glycogen levels are to be very significantly affected. The initial response to extract injection was an elevation of the blood sugar to normal or even supernormal levels. Although glycogenesis might have occurred under the influence of the extracts administered in the latter case, continued storage of liver glycogen was prevented because of its immediate utilization for the purpose of raising the blood sugar level.

Attention should be called to the fact that in our series, hypophysectomy did not result in such precipitate drops in glycogen content of muscle as were seen in the liver. Moreover, no amount of extract served to completely replace muscle glycogen, although the liver glycogen values were elevated to supernormal levels. Thus glycogen storage in muscle would appear to be less subject to cortico-adrenal influence than the process of neoglycogenesis in the liver. The markedly beneficial effects of cortical extracts on muscle activity, and possibly carbohydrate utilization, should nevertheless be kept in mind. Long and Katzin (1938) and also Russel and Craig (1938) have recently published brief reports of findings similar to our own.

These experiments, as well as those of numerous workers noted above, support the hypothesis which has been advanced in an extended series of publications from this laboratory, carried out over many years past, emphasizing the regulatory control of the adrenal cortex on carbohydrate metabolism. The conclusion was drawn nine years ago by one of us that the adrenal cortex appeared to be "concerned with the storage and utilization of carbohydrates" (Britton, 1930). In view of the recent findings of Long and his associates on neoglycogenesis, it might also be mentioned that "some phases of protein metabolism" (*ibid.*) were also thought at that time to be regulated through cortico-adrenal activities. Through the years we have shown that in the absence of the adrenals there is a relative inability to form glycogen; that death occurs after adrenalectomy primarily from carbohydrate exhaustion; and that the cortical hormone is able to raise remarkably the blood sugar and also liver and

muscle glycogen in both normal and adrenalectomized animals (see particularly Britton and Silvette, 1932, 1934, 1937). Even when given by mouth the hormone influences very favorably carbohydrate metabolism in the body (Britton, Flippin and Silvette, 1931).

The present experiments demonstrate that the severe carbohydrate deficiencies which occur in hypophysectomized animals are remedied by the hormone of the adrenal cortex. The carbohydrate content of the body (or glucose and glycogen in blood, liver and muscle) would appear indeed to be almost doubled under optimal conditions by the action of the cortical hormone.

SUMMARY

The inability of hypophysectomized animals to maintain normal carbohydrate levels over short periods of time (12 to 24 hrs.) appears to be explicable on the basis of cortico-adrenal insufficiency.

Administration of highly purified extracts of the adrenal cortex has been shown to be effective in maintaining normal or supernormal blood sugar and liver glycogen levels in hypophysectomized rats. Muscle glycogen also may be kept by the extract at approximately normal values.

Small amounts of extract, e.g., 6 cc. daily, readily produced elevations of blood sugar, and amounts totalling 24–30 cc. daily given in small, frequent doses influence markedly the storage of liver glycogen.

Cortico-adrenal extract was further observed to restore normal blood glucose and liver glycogen values in hypophysectomized rats which were carbohydrate-depleted in a 12-hour fast, before injections were begun.

The cortico-adrenal extracts used were found to be adrenalin and choline free. Differential effects on the blood glucose of adrenalin and cortico-adrenal extract—the action of the latter much more prolonged—were also shown.

The evidence is in keeping with that published nearly a decade ago from this laboratory, and substantiated in numerous papers since, showing specific involvement of the cortico-adrenal tissues in the regulation of carbohydrate metabolism.

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INFLUENCE OF INSULIN ON PROTEIN METABOLISM AS MEASURED BY THE NITROGEN BALANCE

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The protein sparing action of insulin in the diabetic organism is well known. Janney and Shapiro (1) found in normal subjects (human) as well that insulin and glucose also caused a greater sparing of protein than glucose alone. Mirsky has recently reported (2) that insulin has a similar protein sparing action in the nephrectomized dog. Preliminary to a study of the mechanism of this protein sparing action Janney's experiment has been repeated and his results confirmed in the rat.

EXPERIMENTAL. The influence of insulin upon the nitrogen balance of albino rats receiving an adequate diet was determined. The diet was composed of powdered commercial casein 20, potato starch 41, dried powdered medicinal strain brewer's yeast (Anheuser Busch) 10, Osborne and Mendel's standard salt mixture (The Harris Laboratories) 4, cod-liver oil 10 and lard 15. Except in one case (expt. 3), where the food intake was limited, the diet and distilled water were allowed ad libitum.

The rats and the food cups were weighed every second day when the urine and stool collections were made. The latter were collected in dilute copper sulfate solution to prevent the loss of ammonia and dissolved in concentrated sulfuric acid. Aliquots of this were used for the determination of total nitrogen by the macro-Kjeldahl method. A single batch of very carefully mixed food was used for all of the experiments. Three samples of the food were used for nitrogen determinations. The average forms the basis of our calculations. The individual determinations varied considerably due no doubt to lack of complete homogeneity of the diet. Should the figure we have used not represent the true nitrogen content of the diet this can have no influence upon the variation of the nitrogen balance in a given experiment which is what we are interested in.

Regular insulin in a strength of forty units per cubic centimeter was used. When 4 units per day were given each rat received it in a single dose. Ten units were given in two doses of five units each at the ends of the day.

RESULTS. In table 1 are summarized the data on three male rats. The variability of the figures for the two day intervals, probably due to incomplete urine collections and variations in the passage of fecal material,

TABLE 1

WEIGHT OF RAT	WEIGHT CHANGE IN PERIOD	PERIOD	UNITS INSULIN PER DAY	FOOD INTAKE PER DAY	AVERAGE NITROGEN		
					Intake per day	Excretion per day	Retained per day
Experiment 1							
gm.	*gm.*	*days*		*gm.*	*mgm.*	*mgm.*	*mgm.*
322	0	12		10.8	367	342	25
315	−6	4	4	11.5	391	257	134
313	−2	12		9.7	310	209	101
315	2	6	4	10.7	364	103	261
325	10	10		11.0	374	196	178
329	4	10	10	11.7	398	147	251
320	−9	8		9.7	310	184	126
329	9	12	10	11.6	394	118	276
317	−12	8		9.7	310	178	132
Experiment 2							
335	2	12		12.1	411	379	32
343	8	4	4	11.8	401	308	71
343	0	12		10.9	371	309	61
348	5	6	4	11.7	388	134	254
355	7	10		11.9	405	184	221
363	8	10	10	12.9	439	167	272
344	−19	8		10.2	347	293	54
348	4	6	4	11.7	388	163	225
355	6	8		12.0	408	194	214
365	10	10	10	12.8	435	151	284
350	−15	10		10.6	378	170	208
Experiment 3							
360	−23	12		10.0	340	360	−20
337	0	4	4	10.0	340	260	80
337	−9	12		10.0	340	275	65
328	+2	6	4	10.0	340	101	239
330	+1	10		10.0	340	194	146
329	−6	10	10	10.0	340	119	221
323	−4	8		10.0	340	189	151

has led us to consider only the period averages where the error due to these factors would be minimized. In all cases insulin affected the nitrogen balance so that extra nitrogen was retained in the body. Although the insulin usually caused a very slight increase in the food intake this could

not have been a factor in the nitrogen retention for not only were these increases in food intake too small but a fixed food intake gave the same result (expt. 3). The nitrogen retained includes of course that lost in the hair which in rats of this size is considerable. However, it would be most unreasonable to suppose that the amount of nitrogen disposed of in this way might be altered by the action of insulin.

DISCUSSION. Janney (1) believes that in its protein sparing action insulin functions by aiding protein synthesis. Mirsky (2) interprets his data chiefly in the same manner and suggests that insulin aids protein synthesis by increasing the rate of amino acid utilization for this purpose. Janney on the other hand is of the opinion that protein sparing by carbohydrate may be an expression of protein synthesis from carbohydrate metabolites and that insulin aids protein synthesis from carbohydrate by providing necessary metabolites. It is of interest in this connection that the amino acids, the inclusion of which in the diet is dispensable for normal growth (3), and hence presumably may be synthesized by the mammalian organism, are largely those which may yield glucose in their metabolism (4). The influence of glucose and of extra insulin upon the formation of these acids such as glycine to form hippuric acid when benzoic acid is fed should be examined.

SUMMARY

Insulin exerts a definite protein sparing power in albino rats upon an adequate diet. Whether the food intake is fixed or the diet is supplied ad libitum the nitrogen balance is uniformly made more positive by insulin.

We wish to thank Eli Lilly and Company for the supplies of insulin used in this study.

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INCREASED SPONTANEOUS ACTIVITY PRODUCED BY FRONTAL LOBE LESIONS IN CATS

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On the basis of quantitative observations made with cages devised to measure activity, it was reported that removal of the frontal poles of the brain greatly increased the spontaneous activity of monkeys (Richter and Hines, 1938). In order to increase activity these lesions, if made unilaterally, had to include the cortex and at least a tip of the striatum; but if made bilaterally, only the frontal cortex (areas 9, 10, 11, and 12, or only area 9). Further studies were undertaken to determine whether other animals, besides monkeys, become more active after removal of the frontal poles. By means of cages with a revolving drum and cyclometer, it was found that rats deprived of their frontal poles attained activity levels never reached by normals and, in fact, became so active that on several occasions they appeared almost to have reached the point of total exhaustion (Richter and Hawkes, 1939). We have now studied the effect which removal of the frontal poles has on the activity of cats.

METHODS. Cages originally built for monkeys were used in the present work. Since the activity of monkeys consists largely of jumping and climbing, we made the cages very narrow, thus confining the activity to a vertical plane in which it could be measured. Cats also jump and climb, but walking and running make up most of their activity. For this reason a flat cage might have given better results. However, these cages gave sufficiently reliable records for the present purposes. They were made of angle iron frames, 6 ft. long, 6 ft. high, 18 in. wide, covered with a $\frac{1}{2}$ in. wire mesh. A $\frac{1}{4}$ in. rod in the center of the vertical plane of the cage served as an axle for the activity recording mechanism. To this axle was attached a $\frac{1}{8}$ in. flexible steel rod, which in turn was connected to the cat's collar by an 8 in. length of swivel chain. Walking, running, jumping, and climbing movements of the cats around this axle in either direction were registered on two cyclometers attached to the end of the axle. The cyclometers registered every tenth of a revolution.

Every morning the cyclometers were read, the cages were cleaned, and fresh food was provided. The food consisted of ground meat and milk, and was always accessible. We did not measure the food intake.

Preoperative records of the activity were taken for several weeks until the cats reached a fairly constant activity level.

The operative procedure closely resembled that used in monkeys; but since in cats the frontal sinus overhangs the frontal poles, it was necessary to take away part of the sinusoidal one and to fill the opening into the sinus with wax. The ablations included the electrically excitable motor cortex, the premotor cortex, and possibly a small tip of the corpus striatum. The extirpations on the right and left side were performed in two stages, separated usually by an interval of about 60 days.

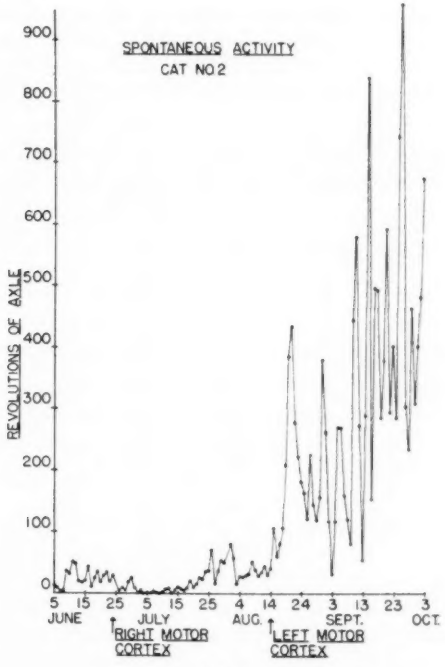

Fig. 1

After the first operation the animals rarely required any special care; however, after the second operation they often had to be fed with a spoon or by a stomach tube. Ten animals were used in these experiments. At autopsy their brains were removed, photographed, and preserved for macroscopic and histological study.

RESULTS. *Effects produced on activity.* After removal of one frontal pole, 9 of the 10 cats became slightly more active, and one showed no change; of the 10 cats with bilateral frontal pole extirpation, 9 showed a marked increase of activity and one a decrease. Six of the 9 cats which

showed increased activity died from 1–16 days postoperatively. Their activity consisted chiefly in pacing back and forth on the floor of the cage and of climbing up and down the sides of the cage from a perch about 3 ft. from the floor. Figure 1 gives the record of one of the animals which became hyperactive after extirpation of the frontal poles. The activity of this animal averaged 25 axle revolutions before the removal of the right frontal pole. Postoperatively the activity decreased to a lower level for the first 30 days, and then increased again, reaching a peak of 80 revolutions at the end of 40 days. The removal of the other pole increased the activity to a much higher level. On two occasions it reached peaks of 840 and 962 revolutions, and averaged approximately 450 revolutions.

TABLE 1

Average daily activity in full revolution of axle

CAT NO.	20 DAYS BEFORE 1ST OPERATION	20 DAYS ON PLATEAU AFTER 1ST OPERATION	PEAK OF ACTIVITY AFTER 1ST OPERATION	FOR SURVIVAL PERIOD OR 20 DAYS ON PLATEAU AFTER 1ST OPERATION	PEAK OF ACTIVITY AFTER 2ND OPERATION	PERIOD OF OBSERVATION IN DAYS AFTER 2ND OPERATION
1	14	119	261	187	478	176 discontinued
2	25	40	80	459	962	52 killed
3	22	25	54	335	589	50 discontinued
4	44	59	104	750	750	2 died
5	20	89	157	221	1,072	15 died
6	22	69	147	502	676	5 discontinued
7	28	50	134	946	2,588	6 died
8	15	34	66	159	1,913	4 died
9	49	60	220	392	697	3 died
10	26	61	110	43	43	1 died
Total.....	265	606	1,333	3,994	9,768	
Average.....	27	61	133	399	977	

Another of these animals became very hyperactive. The activity increased from a daily average of 22 revolutions before the first operation to a level of 69 revolutions 30–50 days afterwards. On the day following the removal of the second pole the activity increased sharply to 480 revolutions and in the next few days to 677 and 634 revolutions. On the fifth day the animal became so sick that we removed it from the cage and discontinued the experiment.

Table 1 summarizes the results for the 10 animals. The activity increased from an average of 27 revolutions for the 20-day period immediately preceding the removal of one pole, to 61 revolutions for the 20-day period taken from the first postoperative plateau, to 399 revolutions for the second postoperative period. One animal reached a peak of 2,588 revolutions on the day before death, which is almost 100 times higher than the preoperative level.

Other changes in behavior. Removal of one frontal pole did not noticeably change the behavior of the cats. Removal of both poles produced marked changes. The cats became abnormally excitable and distractable. Some of them became ravenously hungry almost at once, while others were unable to eat spontaneously and had to be fed for some time. These latter animals which had manifested a disturbed coördination of chewing and swallowing movements learned to eat solid food, such as meat, before they learned to lap milk or water. Similar effects on the feeding responses as well as on activity have been reported (Langworthy and Kolb, 1935; Magoun and Ranson, 1938).

DISCUSSION. The results of these experiments show that cats, like monkeys and rats, become much more active when deprived of the frontal poles of the brain. Some of the cats died either directly or indirectly as a result of the overactivity. The causes of death were difficult to evaluate. In the first few days after operation some of the cats repeatedly butted their heads into the end and corners of the cages, tearing the wound open so that infection resulted. Some of the animals apparently died from over-exhaustion, wearing themselves out by constant activity.

The mechanism responsible for these changes in activity still remains unknown.

SUMMARY

1. Quantitative studies showed that frontal pole extirpation increases the spontaneous activity in cats, even more than in monkeys or rats. Some of the cats reached peaks of activity almost 100 times above their preoperative levels; and furthermore, some of the animals actually died either directly or indirectly from over-exhaustion resulting from the constant activity.

2. Removal of one frontal pole in 10 cats increased the average daily activity from a preoperative level of 27 revolutions to a level of 61 revolutions; subsequent removal of the other pole increased the activity to an average level of 399 revolutions.

3. Some animals became very hyperactive, reaching their highest levels within 48 hours after the removal of the second frontal pole; in others the activity was increased more slowly, not reaching the highest levels for several months.

4. Removal of one pole had very little effect on behavior; subsequent removal of the other pole made the animal ravenous for food, and very excitable and distractable.

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CHANGES IN THE WEIGHT AND WATER CONTENT OF THE UTERUS OF THE NORMAL ADULT RAT[1]

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Recent studies on the immature rat have shown that a prompt increase in uterine tissue water is a constant early index of the action of estrogen (1, 2). Evidence has been obtained that this response can be inhibited by the simultaneous action of progesterone (3). Similar investigations on uterine weight and water content have been extended to the adult rat in anticipation of cyclic changes which would lead to an understanding of normal ovarian function. The fluctuations which were observed were of sufficient magnitude and were so temporally disposed with reference to known events of the cycle that certain conclusions have been reached concerning the time relationships in the normal output of the two ovarian hormones.

METHODS. Normal adult female rats weighing 150 to 200 grams were selected on the basis of normal vaginal cycles. When the stages of the cycle had been accurately determined, groups of 10 to 15 animals were killed with chloroform and their uteri dissected out by cutting the utero-tubal junctions, trimming the mesometria from their uterine attachments and severing the cervices just above their connections with the vagina. Each uterus was split open throughout its entire length and then blotted on absorbent paper and weighed in crucibles or weighing bottles. The values thus obtained consisted of the weights of the entire uteri less the vaginal portion of the cervices and less any fluid which may have been present in the lumina. The uteri were dried in an oven at 110°C., and the decrease in weight taken as their water content. The figures for total and dry uterine weight were expressed in terms of milligrams per 100 grams of body weight.

As it was of prime importance to this study to judge the vaginal smear stages with accuracy, considerable attention was given to this matter. As subsequent results will show, it was highly desirable to know, from the vaginal smear, whether or not an animal in diestrus was about to enter

[1] Aided by a grant from the Rockefeller Foundation administered by Prof. F. L. Hisaw.

[2] Rockefeller Foundation Fellow in the Natural Sciences.

the proestrous stage. It was of aid to this investigation therefore to find that a recognizable smear picture precedes by several hours the appearance of the small round nucleated cells of proestrus. This smear is notable for its sparsity of formed elements and is similar in this respect to the late diestrous smear. It is identified by the presence of loosely formed groups of small oval and rounded cells of varying size admixed with a few leucocytes. As soon as the typical nucleated cells of proestrus appear, this stage tentatively referred to here as "preëstrus," comes to an end. Earlier attempts to time the vaginal stages with precision by means of frequent vaginal smears served to confirm the findings of Emery and Schwabe (4), Ball (5), and of others that such a procedure disturbs the cycle. Recourse was had therefore to daily smears and only on the last day or two were smears made twice daily.

An estimate of the duration of each stage was made on a statistical basis, using the experimental data from several hundred cycles. Each daily smear was assigned a 24 hour rating, and, by dividing the total number of times that a given type of smear was seen by the total number of cycle days, the following approximations were arrived at: cycle length, 109 hours; preëstrus, 4 hours; proestrus, 14 hours; proestrus to estrus (nucleated and cornified cells in equal numbers), 3 hours; estrus (early and late), 25 hours; metestrus, 8 hours; diestrus, 55 hours. These figures are in substantial agreement with the durations given by Long and Evans (6) for the four main stages given above, i.e., proestrus = stage I, early estrus = stage II, late estrus = stage III, and metestrus = stage IV. On the basis of these figures it was usually possible to judge the stages within a narrow margin.

Pseudopregnancy was induced by stimulation of the cervix uteri during the proestrous stage with a strong faradic current, according to the method of Greep and Hisaw (7). In large groups of animals used for other purposes, this procedure has been found to fail only in very rare instances.

Results. The average figures obtained for percentage water, total weight and dry weight during the estrous cycle, and for the first eight days of pseudopregnancy are given in figure 1, and the standard errors of the means are set forth in tables 1 and 2. The percentage water in the uterus reaches a maximum during preëstrus, remains at a high level during proestrus and falls rapidly with the first appearance of cornified cells in the smear. Throughout the whole of estrus, metestrus, and the first day of diestrus, the water content remains at a low level with insignificant fluctuations. The peak in the curve of percentage water during preestrus marks a period of rapid increase in total uterine weight which, however, does not reach a maximum until proestrus. After the subsidence of uterine water, and following a more gradual gradient, the total weight of the uterus falls to a minimum on the first day of diestrus. The

dry weight determinations parallel in a general way the total weights. The maximum dry weight occurs also in proestrus, but the decline thereafter is more gradual and the minimum is reached slightly later. The estrous distention of the uterus, represented diagrammatically at the top of figure 1, becomes fully developed during the period of regression of

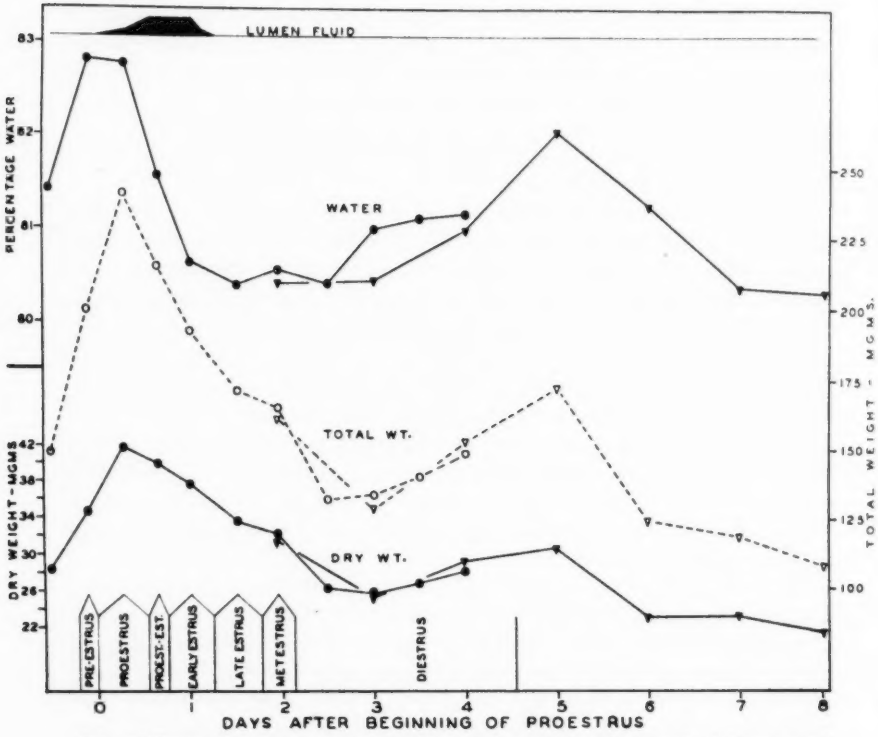

Fig. 1. Percentage water, total weight and dry weight of normal adult rat uteri during various stages of the estrous cycle (circles) and the first eight days of pseudopregnancy (triangles). The weights are in milligrams per 100 grams of body weight. The degree and duration of uterine distention is diagrammed at the top. The vaginal stages and their approximate durations are shown along the abscissa.

uterine tissue water. It is of interest that these fully distended uteri weigh less and contain less tissue water than do uteri before distention takes place.

After the first day of diestrus there is considerable variation from animal to animal in the weight and water content of the uteri. When it is considered that there are considerable individual differences in cycle

length, this variation is to be expected. Some animals on the second day of diestrus are approaching their next estrus, while others at the same stage may not reach the estrous phase for another two days. For this reason, the figures given for the latter part of diestrus are inaccurate as they are average determinations on animals in slightly different physio-

TABLE 1

STAGE	HOURS AFTER BEGINNING OF PROESTRUS	NUMBER OF RATS	BODY WEIGHT, AVERAGE	UTERUS					
				Total weight, mgm. per 100 grams body weight		Dry weight, mgm. per 100 grams body weight		Percentage water	
				Av.	S. E.	Av.	S. E.	Av.	S. E.
Preëstrus	—	7	177	200.9	±7.5	34.6	±1.39	82.82	±0.33
Proestrus	0	5	173	242.2	±10.9	41.7	±2.45	82.77	±0.35
Proestrus to estrus	16	3	167	215.9	±8.4	39.8	±1.65	81.56	±0.09
Estrus-early	24	6	174	193.2	±6.4	37.6	±1.22	80.62	±0.18
Estrus-late	36	7	173	172.2	±10.7	34.0	±2.12	80.37	±0.17
Metestrus	48	4	188	166.0	±13.0	32.5	±3.57	80.66	±0.20
Diestrus	60	6	178	132.9	±9.4	26.1	±1.93	80.39	±0.20
Diestrus	72	10	183	134.4	±7.2	25.6	±1.28	80.98	±0.25
Diestrus	84	7	180	141.2	±15.1	26.0	±2.64	81.09	±0.29
Diestrus	96	9	184	149.5	±5.7	28.2	±0.80	81.14	±0.29
Diestrus	108	7	178	152.2	±7.9	28.3	±1.44	81.41	±0.19

TABLE 2

DAYS AFTER PROESTRUS	NUMBER OF RATS	BODY WEIGHT, AVERAGE	UTERUS					
			Total weight, mgm. per 100 grams body weight		Dry weight, mgm. per 100 grams body weight		Percentage water	
			Average	S. E.	Average	S. E.	Average	S. E.
2	4	172	160.2	±22.3	31.4	±4.88	80.40	±0.34
3	6	184	129.3	±5.2	25.3	±1.34	80.42	±0.28
4	6	178	153.2	±6.6	29.2	±1.64	80.96	±0.15
5	5	184	170.3	±11.3	30.7	±2.21	82.04	±0.26
6	4	162	124.1	±9.4	23.2	±1.19	81.19	±0.56
7	6	188	119.3	±5.2	23.3	±1.08	80.35	±0.26
8	4	182	108.8	±3.8	21.5	±1.01	80.28	±0.21

logic stages. Likewise, animals considered to be in the last part of diestrus (108 hours after their last proestrus) are inaccurately placed for this same reason. In figure 1, determinations on these animals are placed at the left for purposes of clarity, and the very first portion of each curve is therefore questionably exact.

Animals whose cervices were stimulated in proestrus yielded sub-

stantially the same figures during the next four days as did animals in the normal cycle. After the third day, a gradual increase in weight and water content occurred, culminating in significantly high values on the fifth day. After the fifth day, a gradual regression, continuing to the eighth day, carried the values to points below those found during the diestrous stage of the normal cycle.

Although the average values plotted in figure 1 show in a general way the course of the changes which take place, it is highly probable that they do not accurately reproduce the sequence of events or the true extent of the changes which actually occur in the individual animal. For example, the peak in water content lasts for but a few hours, and the chances of detecting this event are much fewer than those of determining some other point just before or just after. There is, moreover, some error in timing the stages, and there also may be some individual variation in the temporal association of uterine and vaginal events. Such unavoidable variables would have the general tendency to smooth out the curve, and it is therefore felt that the maxima and minima through which any individual uterus passes during a cycle are more widely separated than is shown here. The fluctuations in water content, as shown by the averages in table 1, range from 80.37 per cent in late estrus to 82.82 per cent in preestrus, a difference of 2.45 per cent. The extremes, however, range from 79.8 to 84.8 per cent for these two stages, a difference of 5.0 per cent. A sufficiently large number of animals show values in the vicinity of these extremes to make it highly probable that these latter figures would more nearly represent the individual change than does the composite picture of figure 1.

This brief rise in water content has not heretofore been detected, and this is probably due to the fact that slower changes were anticipated. Khayyal and Scott (8) compared estrous with diestrous uteri, and, as can now be readily appreciated, they found no difference in percentage water.

The rapid falling off in uterine tissue water with the onset of vaginal cornification raised the question as to whether this were due to a sudden arrest in estrogen production or to some other influence such as a change inherent in the uterus itself, or to an inhibitory effect of the corpus luteum hormone secreted at this time. To test these alternatives several types of experiment were carried out. First, it was desirable to know the influence of activating the corpora lutea by cervical stimulation. As has already been seen, early pseudopregnancy exerts no modifying influence on the uterine weight or water content of the diestrus. It seemed reasonable, then, to suppose that if this drop in water content took place when the corpora lutea were known to be assuming function, it was more probable that normally they were involved in the reaction during the estrous cycle. Inasmuch as there has been no strict proof that the corpora lutea of the

undisturbed cycle are functional, other possible explanations for this phenomenon were sought.

To investigate the possibility that the uterus itself, having just reacted to estrogen may for a time be refractory to further estrogenic stimuli, experiments involving successive estrogenic stimuli were performed on

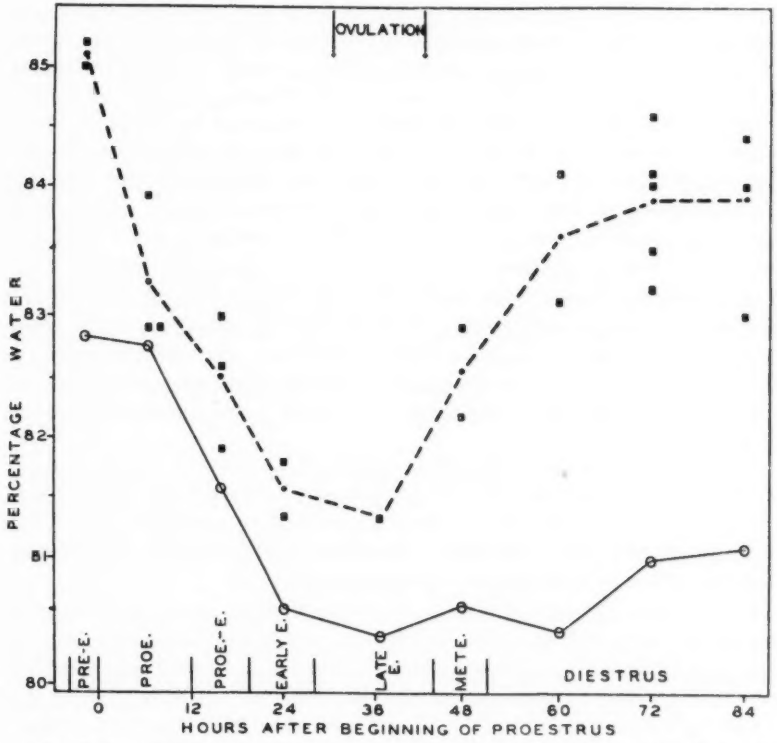

Fig. 2. Average water content of normal adult rat uteri (circles) and of individual animals killed 6 hours after a single injection of 2.0 γ estrodiol (squares) at various stages of the estrous cycle. The dotted line connects the averages of the injected animals. At the top the arrows show the spread in the time of ovulation as determined by Long and Evans.

the immature rat. The prompt rise in uterine tissue water following a single injection of 0.1 γ estradiol has subsided by the end of 48 hours (2). These experiments were repeated, and in addition, one group of animals was given a second injection of 0.1 γ estradiol 42 hours after the first and killed 6 hours later. The averages obtained were as follows: 6 hours after a single injection of 0.1 γ estradiol, weight increase, 69.5

per cent—water content, 85.7 per cent; 48 hours after a single injection, weight increase, 44.8 per cent—water content, 81.6 per cent; 48 hours after a first injection and 6 hours after a second, weight increase, 155 per cent—water content, 86.1 per cent. It would appear then that the uterus itself is not refractory to a second injection of estrogen given before the first has ceased to show effects.

It was further reasoned that if this drop in uterine tissue water just before estrus were due to a sudden arrest in estrogen production, then an injection of estrogen 6 hours prior to autopsy would maintain or restore a high level of tissue water. Consequently, 28 adult animals in various stages of the cycle were injected with 2.0 γ estradiol and killed 6 hours later. The results are shown in figure 2, wherein the solid line is the normal curve of tissue water, and the squares the individual determinations on animals which had received estrogen 6 hours previously. The level for the entire group was somewhat raised by the estrogen, but during proestrus and estrus a strong inhibitory influence is clearly demonstrated. The preëstrous levels were materially elevated indicating that added estrogen can augment the effect of that which is already being secreted by the ovary. As the diestrous stage approached, the uterus regained its ability to respond to injected estrogen. During proestrus and throughout the period of vaginal cornification, a definite inhibitory mechanism is active.

These data indicate that some substance is secreted by the ovary during the period of preovulatory swelling of the follicles, which acts upon the uterus in such a way as to reduce the water content and to inhibit the early action of injected estrogen. Previous experiments (3) have shown that this effect is a property of progesterone.

Discussion. The great change in size which the rat uterus undergoes during the estrous cycle is too well known to require comment except to note that its maximum is reached prior to the appearance of any cornified cells in the vaginal smear and before the occurrence of distention of the uterus by luminal fluid. The further observation that there is a gradual diminution in size after the fifth day of pseudopregnancy suggests that the corpus luteum hormone plays an insignificant rôle in increasing the weight of the uterus. Granting, then, that the major weight increase is a pure estrogen effect, the weight changes alone suggest that the major action of estrogen in the normal cycle is concentrated in a short interval preceding vaginal cornification. Stronger evidence for a limited period of unopposed estrogen action is given by the brief duration of high uterine tissue water. This event, known to follow the injection of estrogen in immature animals by but 6 hours, is highly suggestive of a short period of estrogen release from the ovaries beginning before any vaginal changes are detectable and ceasing during proestrus. The continued increase in total

weight and dry weight after the water has passed its maximum shows that in the normal cycle, as after the injection of estrogen in immature animals, rapid cellular proliferation and increase in total protoplasm is preceded by tissue hydration. The increase in weight and water content during early pseudopregnancy becoming maximal on the fifth day may be correlated with the wave of follicular growth which is also maximal at this time (Swezy, 9).

The sudden drop off in water with the appearance of cornified cells has yielded evidence of two possible mechanisms. In the first place, there may be a sudden and nearly complete cessation of estrogen production, as is suggested also by the progressive loss in total weight, and secondly, there is a concomitant inhibitory influence active in decreasing water content and uterine weight. The data suggest that this inhibitory influence is due to an ovarian secretion having the properties of a corpus luteum hormone. If this be true, this hormone is released from the follicles during preovulatory swelling and continues to act throughout the cornified cell stage. In the normal estrous cycle of the rat, this hormone either ceases to be produced or is secreted in greatly reduced amounts immediately ovulation occurs.

The studies of Hemmingsen (10), of Ball (5), and of others fix the period of most intense heat behavior in early estrus soon after the appearance of cornified cells in the vaginal smear. As a rule, heat begins in proestrus or shortly thereafter and falls in intensity prior to ovulation which takes place toward the end of the period of vaginal cornification (6). Thus, heat behavior is associated in its time relations with the period when the uterus is refractory to injected estrogen; assuming that the above interpretation is correct, it occurs during the period when the corpus luteum hormone is being secreted by the ovary. This observation is of particular interest in view of the findings of Boling, Blandau and Young (11) that heat behavior in the castrated rat primed with estrogen is conditioned by progesterone.

SUMMARY

Determinations on the weight and water content of the uteri of adult rats during closely graded stages of the estrous cycle and during pseudopregnancy show that the uteri reach a maximum size at proestrus and then regress to a minimum on the first day of diestrus. Their water content rises to a maximum before proestrus and then falls abruptly with the first appearance of cornified cells in the vaginal smear. Uteri of pseudopregnant animals follow the same course for the first three days, show an increase in weight and water content on the fifth day, and then regress below the diestrous level. The uteri of animals in various stages of the cycle, given a single injection of 2.0 γ estradiol six hours before death, responded

by a marked increase in tissue water during diestrus and preëstrus. During proestrus and estrus this reaction was strongly inhibited. Evidence is presented showing that this inhibition is due to an ovarian secretion having the properties of a corpus luteum hormone. The events are taken to mean that estrogen acts unopposed during a brief period before proestrus and largely ceases to be produced prior to vaginal cornification. Corpus luteum hormone acts upon the uterus during proestrus and estrus; it is released from ovarian follicles during their preovulatory swelling and ceases to be produced at or shortly after ovulation.

The author gratefully thanks Prof. F. L. Hisaw for his helpful advice and for the facilities of his laboratories while these investigations were in progress.

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EXERCISE IN THE SYMPATHECTOMIZED CAT[1]

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Cannon and his co-workers (1929) have shown that after the entire sympathetic nervous system has been removed cats may continue to lead an existence in the laboratory which is indistinguishable from the normal. Only when the sympathectomized cat is confronted with emergency conditions demanding rapid bodily adjustments, are deficiencies apparent. Thus, the sympathectomized cat presents striking differences from the normal when subjected to extremes of environmental temperature (Sawyer and Schlossberg, 1933) or to anoxemia (Sawyer, Schlossberg and Bright, 1933).

Recent work indicates that the dog, on the other hand, is much less handicapped than the cat by total sympathectomy. On being exposed to high or low temperatures or to anoxemia, the sympathectomized dog shows definite but only slight deviations from the behavior of the unoperated animal (McDonough, 1939; see also Cannon, 1939). Brouha, Cannon and Dill (1936) reported that the dog's capacity to perform severe muscular work is unimpaired by complete sympathectomy. The present study was undertaken to determine the ability of the sympathectomized cat to endure forced physical activity such as work in a treadmill entails.

METHOD. The first treadmill employed was a large hollow wheel, 121 cm. in diameter, mounted on a fixed axle. The wooden sides of the wheel were joined at their circumferences by strong wide-meshed wire net. A strip of heavy rubber, slightly narrower than the net, formed the runway. The treadmill was revolved by means of a crank, the rate of turning being suited to the individual animal. A simple counter attached to the axle registered the number of revolutions. Later a power-driven treadmill for dogs was used.[2]

Preparation of the animals. Healthy cats were selected and tested in the treadmill several times. They were usually not fed for 12 to 18 hours

[1] A preliminary report of this investigation was presented before the American Physiological Society, meeting in Baltimore, Md., April, 1938.

[2] I wish to express my thanks to Dr. D. B. Dill of the Fatigue Laboratory of Harvard University for use of this apparatus and the facilities of the Fatigue Laboratory.

before the experiment. As soon as possible (5 to 15 sec.) after completion of the exercise, the heart rate was counted by means of a stethoscope and an accurate stop watch. The cat was then given meat and milk.

Because cats are not easily induced to work in a treadmill, one of the most difficult problems was their selection and pre-operative training. Sometimes animals which seemed to be learning to run skillfully would, on the third or fourth trial, suddenly stop and refuse to exercise no matter what inducements (food, petting and coaxing) were offered. Only animals which entered the treadmill without great excitement, and ran well in a number of trials on different days, were sympathectomized.

The technique employed for removing the sympathetic system was that described by Cannon *et al.* (1929) except that the superior and inferior cervical ganglia and cervical sympathetic nerves were not removed and the operation was sometimes performed in three, instead of two stages. The animals had recovered from the surgical procedures, had regained their original weight, were eating well, and were apparently normal before they were subjected to another test in the treadmill. The maximal performances in the treadmill and the heart rates after work were compared, after partial and complete sympathectomy, with those that obtained before the operative intervention. Some of the animals were later used in acute experiments to prove the completeness of the sympathetic extirpation.

The effects of subcutaneous injection of atropine sulphate and adrenaline on the running ability and the heart rate of some of the sympathectomized animals were also studied.

Results. Eight cats were completely sympathectomized. Brouha, Cannon and Dill (1936) state that their dogs showed a transient decline in running ability after sympathectomy. This deficiency was attributed to lack of practice, since the severity of the operation prohibited exercise until the normal state was restored. In the present experiments, however, after each stage of the operation, when the animals had recovered sufficiently, some of them were made to run again in the treadmill so that they would need a shorter period of re-training after sympathectomy was completed. Of the 5 cats in which this procedure was followed 2 were able to perform satisfactorily when one thoracic and cervical chain and one set of splanchnic nerves and one adrenal gland represented all that was left of the sympathico-adrenal system. The other 3 animals either did not work so long as during the pre-operative period or worked sporadically and avoided running by sliding along the belt or by other devices. Whether the animals worked well or not, the maximal heart rate (h.r.) after exercise in these partially sympathectomized cats was invariably lower than that recorded in the normal state (see table 1).

Totally sympathectomized cats, when subjected to muscular work, present striking differences from normal animals. In 7 of 8 cases the

TABLE 1

Heart rates per minute

CAT NUMBER	AVERAGE VALUES AFTER EXERCISE		DAYS AFTER SYMPATHECTOMY	COMPLETELY SYMPATHECTOMIZED CATS			
	Normal cats	Cats with 1 thoracic, both abdominal chains and 1 adrenal removed		A	B Atropine sulphate (0.5 to 1.0 mgm. subcutaneously)		C
				After exercise	Before exercise	After exercise or emotion	Adrenaline (0 02 to 0.04 per kgm. subcutaneously) After exercise
1	224	212	14	160			216
			15	172			224
			16	164			216
			18		132	152 180	
			39	160			
			45	180			
			46		144	184	
			58	176			220
			60	Acute experiment—see protocol. H.r. constant			
2	226		15	112 72			
			24	140			
			75		164	196	
3	260		22	220			
			31	208			
			92	204			No h.r. taken
			94		156	192	
4	264	188	15	56			
			18	132			
			21		154	168	
			22	68			
			23				172
			26	144			188
			29				176
			30		152	176	
			40	156			
			41	Both vagi cut aseptically			
			44		120	120	
5	276	220	9	188			
			10	200			
			11	228			
			12	208			
			46		160	212	
			50	Acute experiment. H.r. constant			

TABLE 1—*Concluded*

CAT NUMBER	AVERAGE VALUES AFTER EXERCISE		DAYS AFTER SYMPATHECTOMY	COMPLETELY SYMPATHECTOMIZED CATS			
	Normal cats	Cats with 1 thoracic, both abdominal chains and 1 adrenal removed		A	B Atropine sulphate (0.5 to 1.0 mgm. subcutaneously)		C
				After exercise	Before exercise	After exercise or emotion	Adrenaline (0.02 to 0.04 per kgm. subcutaneously) After exercise
6	211	176	7		124	132	
			8	138			
			9		128	144	
			10	150			
			13		144	156	
			14		132	150	
			15	150			
			16	148			
			18	152	130	152	
			20	152			
			27	172			
			28		144	164	
			30	180	144	176	
			55	196			
			60		188	256	
			62	256			
			64	Branches of left vagus to heart cut			
			68	260			
			76	Right vagus cut below recurrent laryngeal nerve			
			80	196	Evidence of sympathetic regeneration		
7	248	224	15	156			
			19	180			
			22	164			228
			27				212
			28	160			
			30		160	168	
			40	Acute experiment. H.r. constant			

operated animals were unable to equal their pre-operative records. (The eighth cat, not recorded in table 1, is discussed below under the question of regrowth.) The degree of deficiency varied, but in all animals a definite decrease in the running ability was obvious. All but one animal (cat 6, see "regrowth" below) retained this impaired ability throughout the experiment. Commonly an amount of exercise, less than that which in the normal state had little effect, caused, after sympathectomy, extreme exhaustion.

The h.r. of the totally sympathectomized cat after exercise averaged about 30 per cent lower than that during the control period (table 1A). The resting rate was lower than in the normal (Moore and Cannon, 1930).

A protocol will serve to illustrate the typical findings.

Protocol of cat 1—Gray ♀; 3.4 kgm. *Pre-operative period.* Resting h.r., 92. Cat ran well on several occasions on different days in circular treadmill. The mill was rotated about 100 revolutions in 4 minutes. The cat endured this amount of work without noticeable ill effects. Average h.r. after work, 224.

10/26/37. Left thoracic chain from stellate to ganglion beneath dome of diaphragm removed. Left splanchnics cut in chest. Weight, 3.4 kgm.

11/15/37. Weight, 3.5 kgm. Both abdominal sympathetic chains removed from diaphragm to below sacral promontory. Left adrenal gland extirpated but right splanchnics not cut in the abdomen.

11/26/37. Cat well. Weight, 3.5 kgm. Ran for 140 revolutions of the treadmill in 5 minutes. Does not seem unusually tired by the work though it was more intense than before the operations. H.r. after work, 212.

11/29/37. Right thoracic chain removed. Right splanchnics cut in chest.

11/30/37. Incident to a slight struggle while being removed from the cage, the cat began to breathe rapidly and shallowly, went into tonic and clonic convulsions and became limp. She recovered activity but panted violently for about 10 minutes thereafter.

12/13/37. Cat looks well. Resting h.r., 72. In treadmill ran for a total of 89 revolutions; began to show signs of fatigue soon after the beginning of the work. Cat cried throughout the exercise and after 5 minutes, 30 seconds, collapsed. The mill was promptly stopped. H.r. was 160. The cat lay motionless for 8 minutes, breathing rapidly. Cat eventually arose and walked away shakily. This behavior is in decided contrast to that prior to sympathectomy. Forty-five minutes later adrenaline (0.04 mgm. per kgm.) was injected subcutaneously. Five minutes after injection the cat was forced to run in the treadmill. This time the animal ran steadily and rapidly and for a longer time than on its first trial. H.r. was 216. When cat was removed from treadmill it did not appear tired and tried to run away.

12/14/37. Cat ran well for 2 minutes (50 revolutions); then began to slow up and tire noticeably. Cat ran for 15 more rotations but could not go longer. H.r. 172. Lay on her side motionless for more than 4 minutes. After a short rest, adrenaline was injected and the cat was able to run for 6 minutes at a rapid pace and showed none of the signs of exhaustion noticeable on the first run. H.r., 224.

12/15/37. Cat exhausted after short period in treadmill. H.r., 164. Adrenaline again enabled animal to run longer without becoming fatigued. H.r., 216.

12/17/37. 1 mgm. atropine sulphate injected subcutaneously. H.r. constant at 132 while cat was resting quietly. When she was put into the treadmill the h.r. was 152 before any work was performed. Cat exhausted after 3 minutes' running. H.r., 180.

1/5/38. Weight, 3.6 kgm. Cat ran for less than a minute and again showed typical signs of exhaustion.

1/7/38. After a minute's work the cat was exhausted. H.r., 160.

1/13/38. Little work. Cat tired. H.r., 180.

1/14/38. 1 mgm. atropine sulphate. H.r. when quiet, 144. After 2 minutes 30 seconds' work, cat was fatigued. H.r., 184.

1/25/38. Cat fatigued after 2 minutes of moderate work.

1/26/38. Cat exhausted by 1 minute 30 seconds of work. H.r., 176. After adrenaline, cat ran rapidly for 5 minutes. Not tired. H.r., 220.

1/28/38. Acute experiment. Urethane anesthesia. Both vagi cut in neck. Left carotid artery cannulated and blood pressure and h.r. recorded by mercury manometer on a rapidly moving kymograph. Central end of right sciatic stimulated with a strong tetanizing current. An increase of the h.r. of only 2 to 4 beats per minute was recorded, indicating that the sympathectomy was complete and that no regrowth had occurred.

Action of atropine on sympathectomized cats. Although sympathectomized cats are unable to work in a treadmill as well as normal animals a marked cardiac acceleration still occurs. This is ascribable to a decrease of vagal restraint. But even when the effects of the vagal fibers are quite eliminated (as in the completely denervated heart), the heart has a rate which is exceeded in sympathectomized cats subjected to even a small amount of exercise (table 1B). Hence even complete suppression of the vagal cardioinhibitor fibers is insufficient to explain the magnitude of the cardiac acceleration observed in sympathectomized cats. The findings of Jourdan and Nowak (1934) and Brouha, Cannon and Dill (1936) that there are cardiac accelerators in the dog's vagus trunk led to a search for similar fibers in the cat.

Atropine sulphate (0.5 to 1.0 mgm.), injected subcutaneously, caused cardiac acceleration in sympathectomized cats (by paralyzing vagal cardioinhibitors). Table 1B summarizes the observations on 7 cats in which exercise or emotion produced a further augmentation of the heart rate, which in one case was as great as 52 beats per minute. This added speeding after atropine is not due to temperature changes or to the action of metabolites since in some cases the excitement at the start of the running brought it on. Nor can it be attributed to adrenaline or sympathin since these cannot be discharged. It is probably due to accelerator fibers in the vagus trunk (not removed by sympathectomy and the endings of which are not paralyzed by atropine), which are brought into play during exercise or emotion. To test this hypothesis, the right vagus was cut below the recurrent laryngeal, and the left vagus was sectioned in the neck in cat 4. The heart was thus completely denervated. Several days after recovery from the effects of the operation the heart rate was constant at 120 per minute. Atropine now produced no acceleration. Exercise or emotion after atropine likewise elicited no augmentation of the rate. Since in some of the other cats, with vagi cut in an acute final experiment, afferent stimulation revealed no acceleration of the heart, it may be concluded that there was no regrowth of sympathetic fibers (Bacq and Dworkin, 1930) and that the faster pulse produced by work or emotion in the sympathectomized and atropinized animal is due to accessory vagal accelerators.[3]

[3] Some unpublished observations on cats by Doctors Nowak and Maes, made in this Laboratory, point to the same conclusion.

Atropine had no effect on the running ability of sympathectomized cats.

Action of adrenaline on sympathectomized cats. Since removal of the sympathico-adrenal system was accompanied by profound reduction in the cat's ability to work, it seemed possible that the injection of adrenaline might increase that ability or perhaps restore it to normal. Adrenalin chloride (Parke, Davis), in doses of 0.02 to 0.04 mgm. per kgm., was injected subcutaneously into a hind limb of 4 cats on several occasions. The animal was then put into the treadmill. The heart rate accelerated shortly after the injection, but 4 to 6 minutes later it was usually about 100 beats per minute. At this point the treadmill was started. The performance of cats 1 and 4 after adrenaline was strikingly different from that before administration of the drug. On December 14, for instance, cat 1 could run only 2 minutes without becoming exhausted (see protocol). Shortly after this trial, when adrenaline was injected, the cat ran for 6 minutes at a faster speed without exhibiting the signs of distress evident before the injection. While being removed from the treadmill the cat tried to run away and refused to lie quietly as it did in the first experience. The respiratory rate was less increased and the heart rate was faster after adrenaline than in the earlier trial (table 1C). Cat 4 also worked much better and longer, and in one instance ran in the treadmill for 10 minutes, whereas before adrenaline as little as 2 minutes' work caused great exhaustion and distress.

The other 2 cats showed no difference in the quality or quantity of work performed after adrenaline injection.

The question of regrowth. Some observations on 2 cats are significant for possible regeneration of sympathetic fibers. Cat 6, which, as long as 30 days after the last operation was unable to run for more than 5 minutes without becoming fatigued, began to show an ability to run longer and longer without signs of exhaustion. Simultaneously there was a much greater cardiac acceleration. Thus, 60 days after sympathectomy was completed this cat's heart rate after work was 256 per minute. Even when both vagi were cut exercise still caused an increase to 196 per minute. The great rise in the rate, therefore, could not have been entirely due to vagal accelerators and must represent regeneration of some sympathetic fibers. Again, cat 8 ran as well after sympathectomy as in the control period. This discrepant instance can best be explained by some sympathetic regrowth, since the heart rate after atropine when the cat was quiet (260 per minute), is much higher than in completely sympathectomized animals (Moore and Cannon, 1930). In these cats, regrowth of sympathetic fibers to the adrenal medulla probably occurred (Bacq and Dworkin, 1930), though it is possible that a sympathetic nervous connection to the heart might have been re-established by the regenerated preganglionic fibers making synapses in the inferior cervical ganglion.

DISCUSSION. The evidence here presented that the sympathetic division of the autonomic nervous system plays a significant rôle in the cat's adjustments to physical exertion is three-fold. 1. Exclusion of sympathetic nerve impulses results in a marked impairment of the animal's working ability. 2. This deficiency is present for as long as 3 months, provided there is no sympathetic regeneration. When there is evidence that regrowth has occurred the animal partially regains its ability to run in a treadmill. 3. Injection of adrenaline in small doses may markedly improve the sympathectomized cat's performance.

The defects of sympathectomized cats when required to run are in striking contrast to the dog's unimpaired capacity for vigorous, spontaneous and long-continued activity which Brouha, Cannon and Dill (1936) have reported. Possible reasons for this vastly different behavior of the two species may now be considered. Evidence has been adduced that the cat's vagus trunk contains cardio-accelerator fibers (see p. 176). Jourdan and Nowak (1934) demonstrated such fibers in the dog, and Brouha, Cannon and Dill (1936) proved them to be partially responsible for the cardiac acceleration of the sympathectomized dog during exercise. Apparently the species difference cannot be explained on the basis of the cardiac acceleration attainable, since in both cats and dogs the maximal h.r. is reduced about 30 per cent after the operation. Sometimes sympathectomized cats faint if they become greatly excited and struggle, an observation first reported by Freeman and Rosenblueth (1931). These workers recorded a marked fall of the blood pressure of sympathectomized cats concomitant with struggling. On the other hand, only a slight and transient blood-pressure reduction occurs when dogs deprived of their sympathico-adrenal systems exert energetic movements (Pinkston, Partington and Rosenblueth, 1936). This difference in the vascular response of the two species during vigorous activity may at least partially explain the dissimilar findings in their respective capacities for labor after sympathectomy. Further, McDonough (1939) points out that the sympathectomized dog's ability to endure unfavorable environmental conditions may be attributed "to the numerous accessory physiological devices, not under sympathetic control, that are possessed by the dog as a running animal—larger lung and heart per kilogram of body weight, greater blood volume, higher hemoglobin content, abundant salivary flow and a long tongue for heat loss."

The beneficial action which adrenaline may have in dispelling fatigue is confirmatory of the work of Campos, Cannon, Lundin and Walker (1929) on dogs; but the present study offers no further clue as to the possible mechanism of the drug's effectiveness which these authors discuss.

These experiments bring further evidence to support Cannon's view of the importance of the sympathico-adrenal system in the maintenance of homeostasis.

SUMMARY AND CONCLUSIONS

1. Completely sympathectomized cats are able to run much less rapidly and for much shorter periods without becoming fatigued than before the operation (protocol, cat 1). Apparent regrowth of some sympathetic fibers (probably to the adrenal medulla) restores the running ability to some extent (see "regrowth," p. 177).

2. Injected adrenaline may improve the running ability of a sympathectomized cat (p. 177 and protocol, cat 1).

3. The cardiac acceleration after exercise is about 30 per cent less in sympathectomized than in normal cats (table 1A).

4. A cardiac acceleration greater than that caused by removal of vagal inhibition occurs in sympathectomized cats after exercise or emotion (table 1B); this is attributed to accelerators in the vagus trunk (p. 176).

5. Possible reasons for the difference in behavior of the sympathectomized cat and dog during exercise are discussed.

I wish to thank Dr. W. B. Cannon for suggesting the problem and for his interest and helpful advice during the course of this study.

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KINETICS OF CHOLINESTERASE IN BLOOD AND SPINAL FLUID[1]

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Studies of hydrolysis of acetylcholine by the blood cholinesterase have been made by various biological and chemical methods (Ammon, 1933; Glick, 1937; Hicks and Mackay, 1938; Minz, 1932). Similar investigations have been carried out on cholinesterase of body tissues (Marnay and Nachmanson, 1937). There is little work, however, to show that cerebrospinal fluid has an hydrolytic effect on acetylcholine. Stedman, Stedman and White (1933) could not detect cholinesterase in the spinal fluid. Altenburger (1937), on the other hand, using the frog's rectus muscle as an indicator, found spinal fluid to hydrolyze acetylcholine at a rate 1/250 that of the blood.

In this investigation the cholinesterase activities of human blood sera and cerebrospinal fluids were determined by the ear method (Bender, 1938a). The method developed was based on the principle that denervated muscles were sensitive and readily contracted in the presence of small quantities of acetylcholine—a "nicotine" effect of acetylcholine.

METHOD. Cats were used for these experiments. The facial nerve was cut or avulsed aseptically at its exit from the facial canal. After a period allowed for degeneration of nerve endings, usually 4 to 14 days, the denervated facial muscles responded to intravenous injections of acetylcholine. So long as the muscles remained denervated, they served as good indicators by contracting in the presence of acetylcholine. Since in most of these cats the facial nerve was avulsed, regeneration did not take place for many weeks, and muscle sensitivity was maintained. For testing, the cats were anesthetized with nembutal. Eserine salicylate, 0.5 mgm. per kilogram of body weight, was injected intramuscularly to inhibit hydrolysis of the injected acetylcholine by the cat's body tissues. Atropine sulfate, 1.0 mgm., was injected intramuscularly to prevent the muscarine effects of the injected acetylcholine. A needle was inserted into one of the leg veins and fixed by a bull dog clamp. One and 2.0 cc. finely graduated syringes were used to inject the substances. The cat was placed with its denervated side of the face and ear upward. To the inner

[1] This investigation has been aided by a grant of the Josiah Macy Jr. Foundation.

and superior border of the auricle, a light straw, 20.0 cm. long, was attached by means of collodion. A beam of light shining on this straw projected a shadow on a calibrated scale or moving film camera. Any

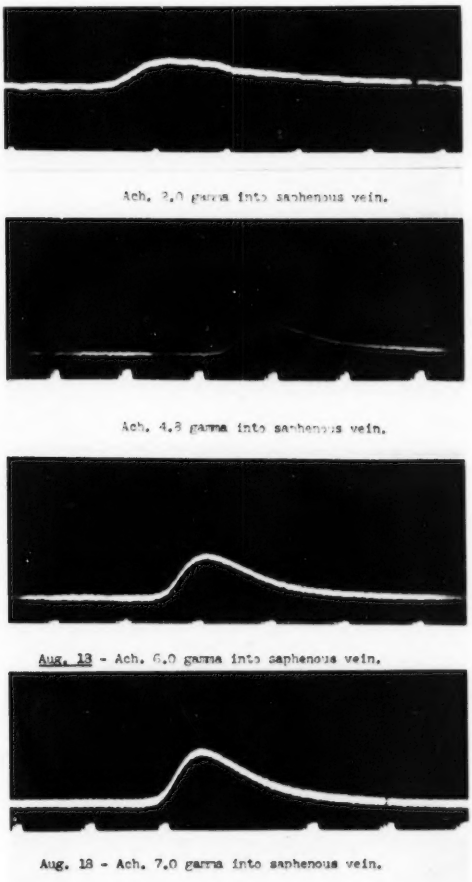

Fig. 1. Optical record of contraction in ear by the intravenous injection of acetylcholine. Cat weighed 3.5 kgm. Time is recorded in five second intervals. Increase in amount of acetylcholine causes increase in muscular contraction.

contraction of the ear muscles was recorded by the shadow on the scale or on the camera lens (fig. 1). The minimal or threshold dose of acetylcholine was that dose which produced a movement of the ear muscles corresponding to the smallest unit on the scale. This smallest unit was a

distance of 1.0 mm. and represented the threshold response or 1+. The threshold dose varied from 0.05 gamma to 0.15 gamma per kilogram of body weight. The smallest threshold dose per animal was 0.09 gamma. The threshold response in each animal was kept fairly constant by the injection of 0.5 cc. of eserine 1:2000 into the leg vein every thirty minutes. The threshold dose was determined from time to time throughout each experiment. For the occurrence of any small variations in the dose, corrections were made in the calculation of per cent of acetylcholine destroyed during the experiment. In one cat the threshold dose was 0.32 gamma; 0.30 gamma gave no response; 0.34 gamma gave a response slightly greater than the threshold. The contraction in the denervated ear appeared six seconds after the injection into the vein (circulation time).

The temperature of the solution mixtures was that of the room, 26°–30°C. Physiologic saline was used as the diluent for serum, cerebrospinal fluid and substrate. The substrate was acetylcholine hydrochloride, a product of E. Merck & Co.

Individual determinations were conducted by mixing the enzyme with the substrate in a beaker or syringe. At measured intervals, aliquots of the mixture corresponding to percentages of the initial amounts of acetylcholine were injected into the cat's leg vein. The period of hydrolytic action extended from the time the mixture was made until the time of injection into the cat's vein. It was assumed that the hydrolysis was stopped by the eserine circulating in the cat's blood as soon as the mixture was injected.

Results. *Calculation of results.* When the injection of the mixture of serum and substrate yielded a movement of the ear muscle of magnitude greater than one unit, then the amount of unhydrolyzed acetylcholine remaining must have been more than the threshold dose. If the movement of the ear muscle was less than one unit, then the amount of acetylcholine which was left unhydrolyzed was less than the threshold dose. Thus 17 seconds after a 0.9 cc. solution, containing 1.0 gamma of acetylcholine in saline, was mixed with 0.1 cc. of human blood serum, 0.4 cc. of this mixture was injected into the circulation of the cat. This caused a movement in the ear corresponding to 1+ or that produced by the threshold dose of 0.32 gamma. The amount of acetylcholine hydrolyzed was $\frac{0.40 - 0.32}{0.40}$ or 20 per cent. Forty-eight seconds later, which was equivalent to 65 seconds of hydrolytic activity, the remainder of the mixture, that is, 0.6 cc., was injected. This produced no contraction in the ear, which meant that more than $\frac{0.60 - 0.32}{0.60}$ or 47 per cent of acetylcholine was hydrolyzed in 65 seconds. In order to obtain more points for graph plotting, the same proportion of serum and acetylcholine was

used but the period of hydrolysis was changed; at twenty seconds the injection of 0.4 cc. caused less than the threshold response (±), and after fifty-five seconds of hydrolysis, 0.6 cc. caused a 1+ movement in the ear or that produced by 0.32 gamma of acetylcholine. Hence 20 per cent of the acetylcholine was hydrolyzed in seventeen seconds and 47 per cent in fifty-five seconds.

Kinetics of Cholinesterase. 1. *Blood serum as source of enzyme.* *a.* By altering the volume per cent of serum and keeping the concentration of acetylcholine constant, a family of curves was obtained (fig. 2). The ordinate represents the percentage of acetylcholine destroyed, the abscissa the duration of the hydrolysis in seconds. The rate of hydrolysis of

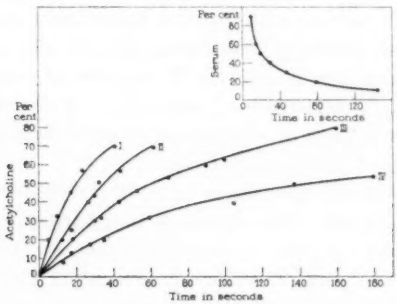

Fig. 2. Cholinesterase activity of different amounts of blood serum of one individual. The initial concentration of acetylcholine solution used was constant—0.0001 per cent. Temperature of room was about the same throughout the experiment. Ordinate—per cent of acetylcholine hydrolyzed. Abscissa—time of hydrolysis in seconds. (I) Serum 30 per cent, (II) serum 20 per cent, (III) 10 per cent, (IV) 5 per cent of total volume of hydrolytic mixture. Inset: Increase in enzyme above a certain concentration does not materially increase the rate of hydrolysis. Ordinate concentration in volume per cent of serum used in the hydrolytic reaction. Abscissa time of hydrolysis in seconds. This curve is representive for 75 per cent of acetylcholine hydrolysis.

acetylcholine seemed to be directly proportional to the enzyme concentration, i.e., the amount of blood serum used in the mixture. As with most enzymes, the amount of change produced by the enzyme was proportional to the enzyme concentration only in the early part of the reaction. The initial concentration of acetylcholine used was the same for all the illustrated curves, 0.0001 per cent or 1.0 gamma per cubic centimeter of mixture. In the inset of figure 2, the ordinate represents the volume per cent of serum used in the mixture and the abscissa the time in seconds it took to hydrolyze a certain per cent (75 per cent) of the initial concentration of acetylcholine. From the type of curve obtained, it is evident that a concentration of cholinesterase, greater than that which exists in

blood serum, would not produce much quicker hydrolysis than that caused by the 90 per cent blood serum used as a source of enzyme in the above experiments.

b. By changing the concentration of the acetylcholine and keeping the amount of blood serum constant, another family of curves was obtained (see fig. 3). The rate of hydrolysis was proportional to the concentration of the reacting molecules at any given time. Evidently, the rate of destruction of acetylcholine depended on the initial concentration of acetylcholine and on the amount of enzyme present in the mixture.

II. *Enzyme activity of different sera.* By keeping the initial concentration of acetylcholine (0.001 per cent or 0.0001 per cent) and the volume of serum (10 per cent of 1 and 2 cc. total volumes) constant, variations in enzyme activity in normal and diseased individuals were obtained. The concentration of acetylcholine was measured but the amount of cholinesterase contained in serum which was 10 per cent of volume of mixture (or 0.1 cc. or 0.2 cc. of serum) was unknown. If the hydrolysis activity differed, it must have been due to the variation of concentration of enzyme in the serum obtained from each individual.

The blood sera tested were taken from thirty different people, most of whom were blood donors. One specimen taken from a patient with myasthenia gravis showed a rate of hydrolysis for acetylcholine which was slightly quicker than that of the same amount of serum taken from the normal people. Two patients with muscular dystrophy had a slightly slower activity curve. In two patients who were moribund, the serum activity was low. The differences, however, are not significant for there are many variations among the sera of normal individuals (Antopol, Tuchman and Schifrin, 1937; Milhorat, 1938).

III. *Cerebrospinal fluid as a source of enzyme.* The activity of cholinesterase in cerebrospinal fluid were similar to that of the enzyme in blood serum but the reaction velocity was much slower. It resembled the activity produced by 1 per cent or 2 per cent volume of serum concentration (fig. 4).

The activity curve was S shaped. The initial velocity was low. This was found on repeated occasions, especially in experiments with low concentration of cerebrospinal fluid. There was a wide variation in the activity of the spinal fluid-enzyme in each of different individuals, and similarly in the activity of dilute blood sera. Any trace of blood, as indicated by red blood cells or xanthochromia, caused a rise in the activity of the enzyme in the cerebrospinal fluid. There was no direct relation between the reaction velocity and the total protein of the cerebrospinal fluid.

Discussion. The results obtained show that diminishing amounts of acetylcholine may remain in contact with blood for several seconds without

being completely destroyed. Working with concentrations of acetylcholine as low as 0.2 gamma per cc. or 0.00002 per cent, it was found that, although most of the acetylcholine was hydrolyzed by the cholinesterase in the first few seconds, there was a remainder which still had "muscarine" and "nicotine" powers. Even when the serum was in concentration of 95 per cent by volume it hydrolyzed 90 per cent of 0.0001 per cent solution of acetylcholine or 0.9 gamma in nine seconds. The remaining 10 per cent of acetylcholine or 0.1 gamma was hydrolyzed at a slower rate in the plateau of the curve, so that traces of the substances must have lingered for longer periods. Smaller initial concentrations of acetylcholine, such as are secreted in the body, that is, 0.01 gamma per cc., would be inactivated even at a slower rate. On the other hand, the higher concen-

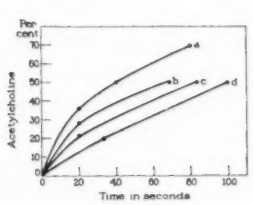

Fig. 3

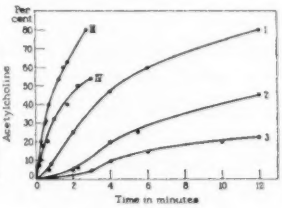

Fig. 4

Fig. 3. Cholinesterase activity of 10 per cent volume of serum on acetylcholine. *a*, 4.0 gammas per cc.; *b*, 2.0 gammas per cc.; *c*, 1.0 gamma per cc., and *d*, 0.5 gamma per cc. Ordinate represents per cent of acetylcholine hydrolyzed; abscissa is the time of hydrolysis in seconds.

Fig. 4. Cholinesterase activity of cerebrospinal fluid (1) 90 per cent, (2) 50 per cent, (3) 25 per cent by volume on acetylcholine 0.0001 per cent compared with the activity of serum 5 per cent and 10 per cent volume of hydrolytic mixture. The ordinate represents per cent of acetylcholine hydrolyzed. The abscissa is the time of hydrolysis in minutes.

tration of enzyme, which may be expected in whole blood as compared with serum, would not materially increase the rate of hydrolysis over that produced by 95 per cent serum. The dynamics of the acetylcholine and cholinesterase reaction appeared to follow the law of mass action. Theoretically this would mean that acetylcholine could hardly be destroyed completely. Infinitesimal amounts would remain unhydrolyzed. Recently Clarke, Raventos, Stedman and Stedman (1938), in their studies on kinetics of cholinesterase of horse serum, came to similar conclusions. Their results showed that the number of acetylcholine molecules hydrolyzed per second per enzyme center was surprisingly small.

In most instances it appeared that the greatest hydrolytic activity of human serum, which was 10 per cent by volume of the mixture or more, occurred in the first twenty seconds or less. Following this, the rate of

hydrolysis diminished. From these and other curves, it is evident that the rate of hydrolysis is rapid in the beginning. There is, however, a possibility that the very initial rate of hydrolysis might be slow for a short period, as inferred from the repeated S shaped curves obtained by the enzymatic action of cerebrospinal fluid and sera diluted one hundred times. The S shaped curves become more conspicuous when the units of the abscissa are contracted. Unfortunately, when the volume of serum used in the hydrolytic mixture is high, the rate of hydrolysis is rapid so that the first ten or fifteen per cent of hydrolysis would occur in the first five seconds. Since in these few seconds it would be difficult to mix the enzyme with substrate and inject into the vein, it would be impossible to measure accurately the initial action of serum. Whether or not S shaped activity curves for serum concentrates of more than one per cent exist, remains to be seen.

If an analogy be permitted at all, between the in vitro experiments reported and the action of cholinesterase on acetylcholine in the body, it appears that a small fraction of the bodily discharged acetylcholine may remain unhydrolyzed. Whatever the source of acetylcholine, whether it be formed at the endings of the autonomic nerves, such as the vagus, or at the synapses of the autonomic ganglia, it is probably discharged at regular intervals. Each amount of acetylcholine discharged, although hydrolyzed for the greater part, would leave an unhydrolyzed amount of a definite small fraction of the original quantity entering the blood stream. If acetylcholine be rhythmically discharged, there is bound to be a fixed concentration level of a fraction of the original amount formed. The quantity of acetylcholine circulating freely in the blood stream may be too small to be detected by the available methods. MacIntosh (1938), working with the leech muscle as an indicator, found acetylcholine in the blood stream of eserinized cats in amounts of the order of 1×10^{-9}. The presence of this substance in the circulating blood, even in lesser quantities, may explain the increase in tonicity and the fibrillations observed in muscles deprived of their nerve supply (Denny-Brown and Pennybacker, 1938).

Any increase in concentration of the circulating acetylcholine may cause contractions in the denervated muscle. Under emotional stress, such as fright, in which the autonomic system is hyperactivated, the amount of acetylcholine produced may be increased. This increased amount of acetylcholine could explain the contraction observed in the denervated facial and ocular muscles of monkeys (Bender, 1938b; Bender and Kennard, 1938). Also if the released acetylcholine reaches the denervated facial muscles quickly, as may be expected under emotional stress, it would not be inactivated as much as if it reached the muscles over a longer period. Feldberg and Krayer (1933) found acetylcholine free in

the coronary sinus of eserinized monkeys. When one considers that the circulation time from the coronary sinus to the facial muscles is about three or four seconds, then only a part or about 60 per cent of acetylcholine would be hydrolyzed leaving approximately 40 per cent of the discharged substance to act on the denervated muscle. In man the circulation time from the coronary sinus to the face is longer than in the monkey and, therefore, more of the acetylcholine would be hydrolyzed leaving a small fraction unhydrolyzed in man.

SUMMARY

1. The kinetics of cholinesterase and small amounts of acetylcholine (0.2 gammas per cc.) were determined by the ear method.

2. The cerebrospinal fluid of man contains cholinesterase. The concentration of the enzyme in the spinal fluid is approximately equivalent to the amount present in 1 or 2 per cent of blood serum.

3. From the in vitro experiments of kinetics of serum cholinesterase and acetylcholine, it is inferred that minute amounts of unhydrolyzed acetylcholine may circulate in the blood stream continually.

It is a pleasure to thank Dr. G. Shwartzman and Dr. H. Sobotka for their valuable criticisms.

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OXYGEN POISONING IN CARDIAC TISSUE[1,2]

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The heart is commonly affected in acute oxygen poisoning. Dautrebande and Haldane (1921) and Bean (1929, 1931) found diminished pulse rates in experimental animals, and Behnke et al. (1935) reported the complete disappearance of the pulse in man, as a result of exposure to oxygen at high barometric pressures. These slowing effects are what might be expected to arise from the failure in reduction of oxyhemoglobin which, as blood color studies (Bean, 1931) have shown, occurs when an animal breathes oxygen at pressures greater than three atmospheres. Such cardiac effects are also in accord with other experimental findings (Gesell, 1923; Campbell, 1929; Hill, 1933) which leave little doubt but that this failure in oxyhemoglobin reduction and the consequent accumulation of carbon dioxide and increased acidity in the tissues is a factor of prime importance in the production of acute oxygen poisoning. It has been demonstrated, however, that oxygen at high pressures may also exert some "direct" toxic influence on non-circulated striated muscle (Bean and Bohr, 1938). Whether a similar "direct" toxic action is operative on the heart to contribute to the cardiac effects of oxygen poisoning in the intact animal, as mentioned above, is a point in question to which the experiments herein reported have been directed.

METHOD. Normally beating frog hearts were isolated and suspended in a compression moist chamber from an open glass cannula inserted into the bulbus arteriosus. This mounting provided a constant equalization of pressure within and outside of the ventricle. To prevent drying, the oxygen used in compression was bubbled through a glass bead humidifier or the heart itself was completely submerged in Ringer solution throughout the experiment. Tin foil electrodes, connected to an induction coil outside the chamber, were placed on the preparation for artificial stimulation in event of failure of the automaticity during exposure to the increased pressure. The temperature was maintained constant except for the slight

[1] Preliminary report—Kongress Bericht II, XVI Internationalen Physiologen Kongress. Zurich, 1938.

[2] These experiments were supported by a grant from the Rockefeller Foundation to Robert Gesell for studies on respiration.

changes (always temporary and usually not more than 2°C.) consequent upon and during the short period (2 min.) of compression and decompression. The gauge pressures employed were 70 and 80 pounds. Photographic recordings were made from which it was possible not only to measure the magnitude of ventricular contraction but also, when supplemented by direct observation, to accurately determine the frequency of the automatic beat of the pace setter. Control experiments were carried out using oxygen at atmospheric pressure and air at 80 pounds' pressure.

Results. A constant finding in all the records was an early increase in the magnitude of ventricular contractions. This increase usually appeared within the first fifteen minutes of exposure to the high oxygen pressure and progressed to a maximum in about two hours, following which there was a gradual diminution. The occurrence of this early increase in contraction amplitude may best be attributed to some initial augmentatory influence of oxygen at high pressure on the contractile processes and it assumes added significance here in view of fact that it often appeared concurrently with an increase in frequency of the automatic beat which of itself would be expected to result in a decreased amplitude of contraction. Any late effects which the high oxygen pressure might have had on the contraction amplitude are masked, in large part at least, by the supervening onset of an adverse influence on the pace setting mechanism. For this reason the records do not offer as conclusive evidence as might be desired concerning the possibility of a late deleterious action of high oxygen on the contractile processes of cardiac muscle. There is, however, some suggestion of such an influence in the records taken in the more prolonged exposures but it would appear that the contractile process of cardiac muscle like that of striated muscle (Bean and Bohr, 1938) may be adversely affected by high oxygen only after very prolonged exposure.

Recognizing that excised frog hearts are subject to a decrease in conductivity (Clark, 1913) and that the use of ventricular rate as an index of pace setter activity demands therefore, especial precautions, supplementary direct observations were made on all preparations wherever an altered rate might be suspected of arising from an altered conductivity. However, even without this added precaution of direct observation, the progressive nature of the recorded alterations in frequency, arising from the continued exposure to high oxygen pressure, is in itself a fairly safe index that such frequency alterations had their site of origin in the pace setter rather than in the conductive mechanism. A diminished conductivity, manifest as A-V block occasionally occurred, not only in those control preparations exposed to air but also in those exposed to oxygen at atmospheric pressure. This condition of block could be relieved by raising the oxygen pressure to the 70 or 80 pounds as used in these experiments. Oxygen at high barometric pressure appears, therefore, to offset the dimin-

ished conductivity of the excised heart (whether it be due to a loss of lipoids (Clark, 1913) or some other cause) and to actually improve conductivity—at least initially. That this improvement was due to the high oxygen pressure rather than to pressure itself is indicated by the finding that a block present in a preparation exposed to air at atmospheric pressure (or submerged in Ringer solution bubbled with air) persisted after compression of the air to 70–80 pounds' gauge pressure and this even though the partial pressure of oxygen at such pressures is slightly over one atmosphere. Although these results indicate an initial improvement in conductivity of excised hearts there is no evidence concerning possible late effects of prolonged exposure on conductivity. Such late effects, whatever they may be, like those on contractility, have been overshadowed in these experiments by a greater susceptibility of the pace setter to the deleterious action of oxygen at high barometric pressure.

The frequency level of the automatic beat of the excised hearts was consistently higher than that obtaining in situ. Contributory to this elevation was the chamber temperature which, being maintained at room temperature (usually 24.5°C.) was higher than that of the heart in situ. Manipulation in mounting the preparations may have been an additional contributing factor.

The frequency of the automatic beat in those hearts exposed to gaseous oxygen (and in those control preparations exposed to air) increased, temporarily, during compression. This was due to the concomitant transient rise in temperature consequent upon compression rather than to the effect of the pressure itself. Such an explanation is substantiated by the absence of any similar alteration in frequency in those preparations submerged in Ringer solution, the temperature of which remained practically constant during compression. The submergence of the preparations in Ringer solution contributed also to the greater stability of beat frequency prior to actual compression. Following this initial frequency alteration consequent upon compression, the heart rate of the test preparations exposed to high oxygen pressure remained relatively constant for periods of from 3 to 6 hours. It then fell rapidly but progressively to complete cessation. Decompression to atmospheric pressure failed to bring about recovery of the automaticity once the heart had stopped but such decompression if begun when the heart had slowed to about fifty per cent of its initial frequency resulted either in a partial recovery or a suspension of any further decrease in rate.

Single shock electrical stimuli applied to those hearts, whose automatic beat had been stopped by, and which were still exposed to, the high oxygen pressure, elicited strong single contractions which occasionally were followed by a short lived return of automaticity. Successive attempts to reëstablish the automatic beat in this manner were attended by a rapidly

progressive shortening of the recovery periods to eventual extinction. In contrast, artificial stimulation applied *after* the pressure had been lowered to atmospheric, resulted in an essentially permanent recovery of automaticity in 75 per cent of the preparations and in the remaining 25 per cent the recovery was of longer duration than that elicited by similar stimulation applied during the maintenance of the high pressure. This contrast in pre- and post-decompression stimulation effects was on the whole more pronounced in those experiments in which the preparations were submerged in Ringer solution and equilibrated with oxygen at 80 pounds.

Records of typical experiments are summarized in the plotted graphs of the accompanying figures. The changes in automatic rhythmicity of the heart induced by exposure to, and by subsequent decompression from, oxygen at 70 pounds' pressure are shown in figure 1. The solid line

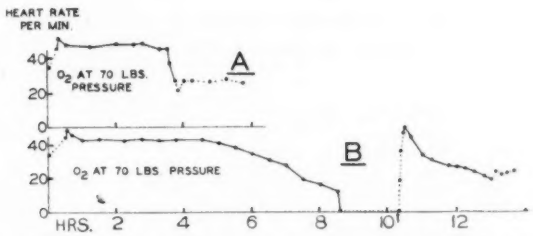

Fig. 1. The effect of oxygen at 70 lbs. gauge pressure on the rate of the automatically beating isolated frog heart. The solid line curve denotes the periods of exposure to oxygen at 70 lbs.; the broken line denotes exposure to oxygen at atmospheric pressure. Arrow on the abscissa indicates application of auxiliary single shock electrical stimulus.

portions represent the frequency of the automatic beat during exposure to 70 pounds, the broken line portions, the frequency at atmospheric pressure. In each of the two experiments represented, there is shown an initial increase in heart rate. Curve *A*, figure 1, shows this increase was maintained with only slight decrease for $3\frac{1}{2}$ hours. The subsequent rapid decrease to about 50 per cent of its initial rate was effectively stopped by decompression to atmospheric pressure but there was no return to the initial frequency. In curve *B*, derived from another heart, the decrease in frequency was more delayed in its onset, less precipitous than in *A*, and continued to complete cessation of beat. Here decompression of itself was ineffective in causing any recovery. However, single shock stimuli applied one hour after cessation of the beat, and after decompression to atmospheric pressure, induced a return of the automaticity to its initial frequency. A second exposure of this recovered preparation to high oxygen resulted in a very much earlier onset of the drop in frequency than did the first exposure.

The subsequent decompression, begun before complete stoppage of the beat, not only checked this fall in rate but actually turned the curve in the rising direction.

Figure 2 illustrates an initial increase and late decrease in frequency, effected by exposure of the automatically beating heart to oxygen at 80 pounds' pressure. With this pressure the decrease to complete cessation was usually more precipitous than that recorded during the 70 pound exposures. But even after $5\frac{1}{2}$ hours of exposure to this higher pressure the contractility of the cardiac muscle was as yet apparently unimpaired since single shock stimuli applied to the heart which had been stopped by, and which was still exposed to the high oxygen pressure, elicited strong contractions. However all attempts to bring about a persistent recovery of automaticity by such stimulation (indicated by the arrows along the abscissae) during the maintenance of the pressure, failed. On the other hand, electrical stimulation applied *after* decompression brought about either a prolonged or a permanent return of the automatic beat. The permanency of this recovery automaticity was directly related to the in-

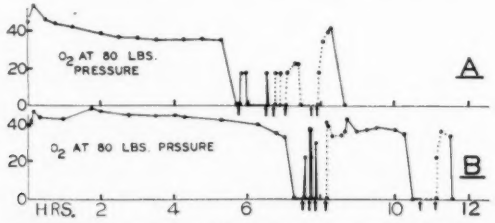

Fig. 2. The effects of oxygen at 80 lbs.' gauge pressure. See legend of figure 1.

terval between the end of decompression and the application of the auxiliary stimulus, and inversely related to the duration of the exposure to the high oxygen pressure. That the effect of high oxygen is not entirely and rapidly reversible is suggested in these records by the progressively earlier onset of the effects in successive exposures (hearts *A* and *B*, fig. 2).

Results typical of those derived from the preparations suspended in Ringer solution are shown in figure 3. The slight temperature rise observed during compression in the exposures to gaseous oxygen was practically eliminated in the equilibrated Ringer solution experiments. This no doubt is one reason why the temporary initial increase in frequency of beat as shown in figures 1 and 2, was never significant in the submerged preparations. The recovery of automatic beat elicited by single shock stimuli applied after decompression is shown in *A*, figure 3. It was found also that reëstablishment of the automatic beat could be accomplished without the auxiliary stimulation, by replacing the Ringer solution in which the heart had been stopped, by fresh Ringer solution equilibrated with oxygen at atmospheric pressure (*B*, fig. 3).

The pace setter automaticity of control preparations mounted in air and exposed to 80 pounds' pressure, and of those controls exposed to oxygen at atmospheric pressure, continued active without any significant alteration in frequency for periods as long as 12 hours.

DISCUSSION. These results indicate that oxygen at high barometric pressure exerts an early augmentatory, and a later deleterious influence on isolated non-circulated cardiac tissue. The delayed deleterious effect on this isolated tissue obviously cannot be attributed to the hemoglobin dysfunction mentioned above which occurs in the intact animal so it may perhaps best be called a "direct" toxic action. The apparent absence of any early "direct" toxic action in these experiments is in accord with the finding of Hill and Macleod (1903) that a frog's heart will beat for more

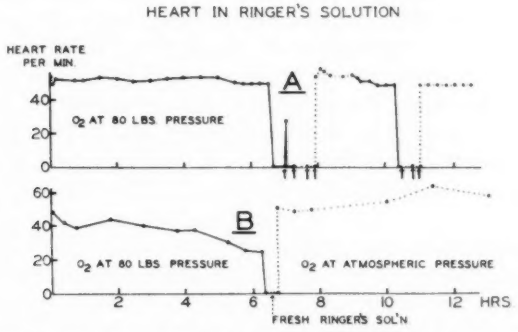

Fig. 3. Heart exposed to oxygen in Ringer's solution. Broken arrow in part *B* marks replacement of Ringer solution equilibrated in oxygen at 80 lbs.' pressure by Ringer solution equilibrated in oxygen at atmospheric pressure. Otherwise legend as that of figure 1.

than an hour exposed to oxygen at fifty atmospheres' pressure. Hill and Macleod found a decrease in carbon dioxide output in warm blooded animals exposed to high oxygen pressure and suggested that such decrease may have been due to a lowered diffusion of carbon dioxide consequent upon the increased density of the compressed oxygen. Theoretically this same factor should have been operative in our non-circulated heart experiments but the results of control experiments in which air was used as the compression medium eliminates the possibility of tissue hypercapnia arising from a decreased diffusion as an explanation of the high oxygen effects recorded in our isolated heart experiments.

It has been suggested (Bean and Haldi, 1932; Bean and Bohr, 1938) that the enzymatic processes of tissue oxidations may be involved in oxygen poisoning. Considerable experimental information has accumulated in support of this view. Massart (1936) found that high oxygen pressure results in a cessation of cellular respiration (as measured by carbon dioxide

output), corresponding to a blockage of cytochrome in the oxidized form and since the cytochrome itself was not damaged by the high oxygen pressure it was concluded that the activity of the cytochrome reducing system was greatly diminished. Librecht and Massart (1937) in further studies on cellular respiration found that high oxygen pressure caused a complete inhibition of the activity of fresh succino-dehydrogenase. We have performed similar experiments on fresh succino-dehydrogenase as obtained from frog striated muscle and preliminary results are in agreement with those of Librecht and Massart. The apparent irreversible effect of high oxygen on this enzyme may explain the cumulative action of successive exposures of the isolated hearts to high oxygen shown in the figures above.

The inhibitory effect of high oxygen on oxidative mechanisms might lead to an accumulation of fixed acids and an increased hydrogen ion concentration of the tissues. Daly and Clark (1920) and Andrus and Carter (1924) in extensive investigations of the effects of altered hydrogen ion concentration in fluids perfused through isolated hearts report a decrease, in rate, in conductivity, and in strength of contraction, as a result of increased acidity. It may be that an increased acidity contributed to our findings in the non-circulated frog hearts. Additional evidence on this point of conjecture might be uncovered by cH determinations of the tissues themselves.

It is not improbable that the "direct" toxic effect of high oxygen observed in isolated heart is a contributing factor to the occurrence of oxygen poisoning in the circulated mammal. However, the very early onset of symptoms of poisoning in intact animals together with the concurrent upset in carbon dioxide carriage, as contrasted with the late onset of the "direct" toxic effect in isolated tissue would seem to indicate that the primary cause of the cardiac effects in oxygen poisoning in the circulated animal is the disturbed hemoglobin function rather than the "direct" toxic action. Both factors, however, would seem to be travelling different roads to a common destination—an increased tissue acidity. The delayed onset of toxic signs in isolated tissue may have been occasioned in part at least by a slower diffusion of oxygen in the non-circulated preparations and perhaps also to the low metabolic rate of frog tissue.

SUMMARY AND CONCLUSIONS

1. Experiments are described in which contractions of isolated non-circulated frog hearts exposed to oxygen at 70 and 80 pounds' gauge pressure were recorded.

2. Oxygen at these pressures caused an initial increase and a slight but very late decrease, in the strength of ventricular contraction; a delayed (3–6 hrs.) slowing in frequency and an eventual complete cessation of automaticity. That the pace setting mechanism was more susceptible to this "direct" deleterious effect of high oxygen than was the contraction

mechanism was shown by the persistence of cardiac muscle irritability to electrical stimulation long after the automatic beat had failed. The conductivity in excised hearts appeared to be initially improved by oxygen at high barometric pressure. Possible late effects on the conductive mechanism were not apparent in these experiments.

3. Decompression to atmospheric pressure begun after the onset of the slowing but before complete cessation of the automatic beat, was frequently followed by either a definite recovery, or a stoppage of any further decrease in frequency. Decompression of itself begun after complete cessation of the automatic beat, invariably failed to bring about any evident recovery.

4. Single shock stimuli applied to the heart after it had been stopped by a maintained pressure failed to induce any persistent recovery of the pace setter though the muscle itself remained irritable. Similar stimulation applied following decompression, however, induced a more or less permanent recovery of automaticity.

5. Recovery of the automaticity of submerged hearts previously stopped by high oxygen pressure in Ringer solution, was induced without auxiliary stimulation by replacing the high oxygen pressure solution by a similar one equilibrated with oxygen at atmospheric pressure.

6. The "direct" toxic influence of high oxygen pressure demonstrated in isolated hearts in all likelihood contributes to the cardiac effects observed in intact animals exposed for long periods, to oxygen at high pressure, but it would seem to be of less significance in such cases than the disturbed carriage of carbon dioxide. This "direct" toxic effect is apparently cumulative and not completely reversible after prolonged exposures.

7. Evidence is cited in support of the possibility that this "direct" toxic action involves a poisoning of respiratory enzymes.

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SEPARATION OF THE RESTING AND ACTIVITY OXYGEN CONSUMPTIONS OF FROG MUSCLE BY MEANS OF SODIUM AZIDE[1]

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Considerable interest is attached to the problem whether the resting rate of respiration of muscle represents simply a low rate of activity of the mechanisms concerned in contraction and recovery or a qualitatively separable system. There are many well-known cases based mainly upon sensitivity to specific chemical inhibitors in which the total maximum respiration can be separated into fractions, an inhibitor-sensitive and an inhibitor-insensitive fraction, (e.g., sea urchin eggs by Runnström, 1928, 1934; grasshopper eggs by Bodine and co-workers, 1934, *et seq*; *Neurospora tetrasperma* by Goddard, 1935, Goddard and Smith, 1938; possibly many mammalian tissues, Dixon and Elliott, 1929, but see Van Heynigen, 1935; etc.). Clark and White (1928) found that the action of electrolytes on heart muscle could be attributed entirely to effects on the "work-metabolism," leaving a resting fraction whose chemistry they postulated to be different from that associated with tension development (but cf. Victor, 1933). More recently Deutsch and Raper (1938) have demonstrated marked differences between the resting and "activity" respirations of salivary gland tissue.

In frog muscle the presence of ample quantities of cytochrome and its oxidase (Keilin, 1925) and the fact that the oxygen consumption could be almost completely inhibited by cyanide (Meyerhof, 1930, p. 17) seemed to settle the question in favor of the presence of only one oxidizing system under ordinary circumstances. However, in an attempt to substitute sodium azide for cyanide as a respiratory inhibitor it became apparent that the oxygen consumption of resting isolated frog muscle was relatively insensitive to this poison. On the other hand the increment in respiration caused by stimulation of the muscle either electrically or chemically was markedly sensitive to azide. It is believed that these observations indicate an essential discontinuity under these experimental conditions be-

[1] A preliminary note on some of this work appeared in Biol. Bull. **75**: 375, 1938.

tween the systems or certain steps in the systems of oxygen consumption in resting as compared with active frog muscle. Reasons for preferring this point of view to an explanation based upon "unsaturation" of the enzyme surfaces will be presented after the experimental data.

METHODS. Oxygen consumption and glycolysis were measured in Fenn differential volumeters with bottle volumes and capillary capacities such that one centimeter deflection on the scale corresponded to a change in volume of 6 to 9 cubic millimeters of gas. It should be noted that this provides sufficient sensitivity to measure satisfactorily the respiration of intact frog muscle in the resting state (25 to 35 cmm./g./hr.) without undue prolongation of the experimental period and without the necessity of using large amounts of tissue. This factor is considered important in these studies. Paired sartorius, semitendinosus, iloefibularis, and tibialis anticus longus muscles were used averaging 200 mgm. per respirometer. The sartorius was employed most frequently. For oxygen consumption measurements the gas space contained air, the CO_2 was absorbed by 10 per cent KOH, and the muscles were suspended in Ringer-phosphate (Stannard, 1938) except for experiments with KCl and caffeine. Several experiments performed in 100 per cent oxygen instead of air indicated that no essential change in the results would ensue, although at the highest rates of metabolism found, for example, in caffeine the control rate of oxygen consumption was limited by O_2 tension in air.

Almost all experiments were doubly controlled, one respirometer serving as a continuous control while the others were arranged so that reagents could be tipped onto the tissue after a preliminary control period. Whenever possible, comparisons were made between paired muscles. All concentrations reported were calculated on the assumption that 80 per cent of the muscle wet weight represented water. In the experiments with caffeine the muscles were first soaked in a large volume of caffeine-Ringer solution and then suspended in this solution in the respirometer. When it was desired to tip KCl solutions the preliminary control period was carried out in the absence of Ringer's solution.

Winter and summer, but not spring frogs have been used. The experiments with summer frogs were carried out at the Marine Biological Laboratories, Woods Hole, Massachusetts. The same apparatus was used, and as far as the particular properties under investigation were concerned the summer frogs at Woods Hole behaved as well if not better than winter frogs in Rochester. Throughout the work the maintenance of a steady base line is important and in all experiments the muscles were dissected the night before use and equilibrated in Ringer's solution at about 5°C. overnight. After this treatment in over 150 determinations at 22–23°C. the resting rate of oxygen consumption averaged 30 ± 5 cmm. O_2/g./hr.

All reagents were diluted with the appropriate Ringer's solution (except when KCl was employed). The pH of the solutions before and after each experiment was checked colorimetrically or against a glass electrode. The temperature was kept constant to ±0.01°C. Approximately half of the experiments were performed at 22°C., the remainder at 23°C.

RESULTS. 1. *Resting muscle.* Keilin (1936) has studied the action of sodium azide on the respiration of yeast and some other catalytic oxidations. He found it to be a potent inhibitor of yeast respiration at acid pH. In general its action seemed similar to that of cyanide, and its lower vapor pressure presents an advantage over cyanide for use in manometric experiments. Hence the first experiments in this study were simply an attempt to substitute azide for cyanide as a respiratory inhibitor. But instead of the expected inhibition M/1000 azide caused 50–75 per cent stimulation of the oxygen consumption of resting muscle. At higher concentrations the stimulation did not appear, but practically no inhibition ever occurred during the first hour of observation. If sufficient time was allowed slow inhibition set in at all concentrations above M/1000.

Since Keilin (1936) had found the inhibitory action on yeast respiration to be markedly decreased by increasing alkalinity, and since the normal pH of the Ringer's solution (7.4) was in the range in which no effect was observable in yeast, it seemed probable that the lack of effect was due to this phenomenon. Hence muscles were equilibrated overnight and studied in Ringer's solutions at pH 6.0 and 4.6. Although the intracellular pH may not have been brought to these values it seems probable that this treatment should have sufficed to demonstrate any marked dependence of the inhibitory action on pH, especially since the changes found in yeast were very marked. Table 1 illustrates the effect of H ion concentration on the action of azide on resting muscle. Although the stimulation appeared to be diminished no marked diminution in the rate of respiration in azide appeared at the more acid reactions. The data presented are for the first hour of the experiment. It should be said that as time progressed the muscles in the acid-Ringer's showed a more rapid fall in the rate of oxygen consumption than controls at pH 7.4. However, increased acidity while not without effect, presented a picture contrasting strongly with that expected on the basis of yeast experiments. Since as will be shown presently, marked inhibition of any increment in respiration over the resting level occurred even at pH 7.4 slow penetration cannot be an explanation for the lack of effect of azide on the resting metabolism.

Figure 1 presents a summary of the experiments on resting muscle. It will be seen that over a ten-thousand fold range of concentrations the oxygen consumption was not inhibited by this poison.

2. *Contracture.* The cause of the stimulatory action on the O_2 uptake of resting muscle in the middle concentration ranges (cf. fig. 1) was found to

be due to a slow contracture. The contracture was measured by means of kymographic records taken on matched muscles placed in azide solutions of the same concentration as that employed in the particular respira-

TABLE 1

Comparison of effects of NaN_3 on O_2 consumption of resting muscle at normal and acid pH

EXPERIMENT	CONCENTRATION AZIDE $M \times 10^{-3}$	pH	O_2 UPTAKE	
			Azide	Control
			cmm./g./hr.	*cmm./g./hr.*
A	4.0	7.4	57	34
	4.0	4.6	30	27
B	4.1	7.4	29	20
	4.1	4.6	27	30
C	2.0	4.6	34	31
D	2.2	6.0	22	26

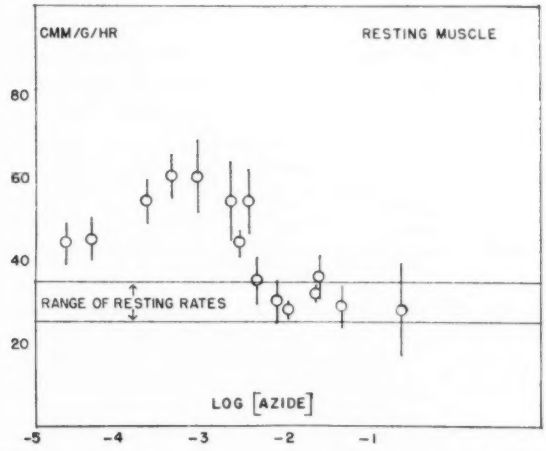

Fig. 1. Oxygen consumption of resting muscle at various concentrations of azide over a ten-thousand-fold range. Concentrations given in this figure and in figures 5 and 7 are in moles per liter. The height of the bars represents the average deviation of observations at each concentration. The range of resting rates was taken from 150 determinations on untreated muscles. The cause of the stimulation in the middle ranges is explained in the text.

tion determination. That chemical contractures in general cause a rise in oxygen consumption is evident from the work of Fenn (1931). Actually the contracture occurred also at the higher concentrations of poison

where no increase in oxygen consumption is apparent in the figure. In fact the contracture at M/10 azide was as rapid and vigorous as that caused by isotonic KCl. But at this concentration of azide no rise in O_2 consumption was evident.

This superficially contradictory result is explained by the fact that sodium azide exerts more than one effect on resting frog muscle. This was best demonstrated by experiments on the anaerobic glycolysis. The glycolysis was measured in the usual manner by displacement of CO_2 from Ringer-bicarbonate solutions equilibrated with CO_2-nitrogen mixtures (Stannard, 1938, for further details). The results of a typical ex-

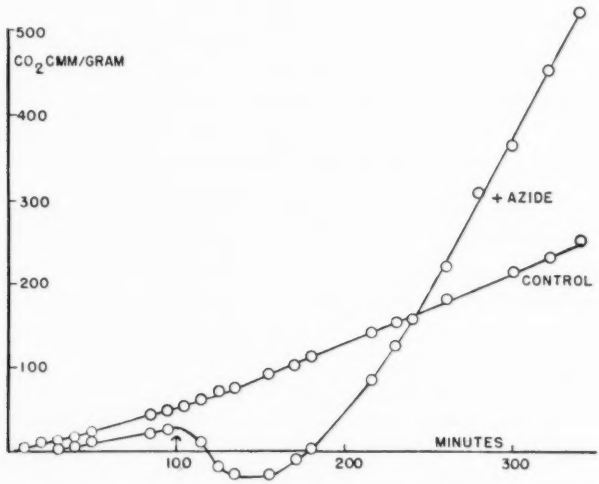

Fig. 2. Anaerobic CO_2 production of resting muscle plotted as total CO_2 against time. The azide-treated pair of muscles started at time 25 minutes and azide was tipped at 100 minutes. Final concentration of azide was 0.0021 M; the medium Ringer-bicarbonate (0.02 N) equilibrated with 5 per cent CO_2 in nitrogen.

periment are shown in figure 2. While the CO_2 displacement proceeded in the control at a rate which increased slightly with time, when azide was tipped onto the experimental tissues there was first an absorption of CO_2 (associated with phosphocreatine breakdown) followed by a greatly augmented rate of CO_2 production (due to increased lactic acid production). This duplicates the picture presented for stimulated muscle by Meyerhof and Lipmann (1930) and for KCl contractures by Tipton (1936). The final rate of lactic acid production was about 8 times the control rate. Calculation (assuming an oxidation quotient of five) shows readily that at the rates of oxygen consumption prevailing at this concentration of azide

(fig. 1) lactic acid should accumulate aerobically. This was borne out by estimations of the pH in aerobic experiments. The buffer power of the Ringer's solution was exhausted after about an hour, and from this point on the pH fell. The final level reached depended, of course, upon the duration of the experiment and the concentration of azide. It was lower the longer the experiment and the higher the concentration. In an average 4 hour experiment at 0.002M the final level was about 0.2 pH unit lower than the control. If left 12 hours, the final level might be 6.8, a fall of 0.6 unit. However, no formal study has been made of the course of this change with time.

It was thus obvious that the azide was producing a contracture accompanied by inceased lactic acid production (or the initiation of lactic acid production) and at the same time preventing oxidation of the lactate produced, the amount of this inhibition depending on the concentration. As the concentration increased, the contracture and dependent lactate formation were actually increased, but the oxidation of the accumulating lactate was completely prevented. At the lowest concentrations the contracture was very small. On this basis the data in figure 1 obtain a complete explanation.

Both the effects on oxygen consumption and the contracture were reversible if the poison was present for limited periods (e.g., 30–40 minutes at M/1000). After longer periods of exposure the muscles did not regain irritability (which was lost during the contracture) nor could the oxygen consumption be increased above the resting level. Yet the initial contractures and oxygen consumption effects occurred within 10 minutes, a much shorter time than the maximum exposure time after which the effects could be reversed. Thus "reversibility" was not simply lack of sufficient time for the full effect.

None of the effects noted above were obtained when equivalent concentrations of sodium chloride were substituted for sodium azide.

3. *Chemical contracture.* To illustrate further the inhibitory effect of azide on the extra oxygen consumption associated with chemical stimulation, experiments were arranged in which isotonic KCl or 0.002M acetylcholine with or without azide could be tipped onto muscles after a preliminary control period. The data are included in table 2. The figures for the treated muscle without azide represent average rates of oxygen consumption. Actually, of course, the rates were very high soon after tipping and fell gradually with time (cf. Fenn 1931). However, the data in the presence of the chemical stimulant *plus azide* represent constant rates of oxygen consumption throughout. Thus there was no increased O_2 uptake in azide-treated muscle. The agreement of the residual rates with those for untreated, resting muscle is within the normal range of variation. Kymograph experiments showed that the poison did not in

any way interfere with the normal contracture caused by these reagents; it simply prevents the increase in oxygen consumption.

4. *Electrical stimulation.* Two respirometers were used with platinum electrodes sealed in at the base so that the muscles could be stimulated while immersed in a small quantity of Ringer's solution. Side-arms were fitted so that azide-Ringer's could be tipped onto the tissues. After a control period for measurement of the resting respiration the muscles could be stimulated and the extra oxygen consumption estimated. The experiment could then be repeated, and when desired azide was tipped onto the muscles a few seconds after stimulation. As illustrated in figure 3 no rise in oxygen consumption followed stimulation when azide was added immediately afterwards. This experiment has been repeated many times with the same result. The particular experiment illustrated was chosen because it was possible to stimulate the muscles twice after tipping the poison with noticeable contraction (observed visually) but no rise in

TABLE 2

Effect of sodium azide on increased oxygen uptake due to chemical contractures

SUBSTANCE ADDED	CONCENTRATION	CONCENTRATION AZIDE $M \times 10^{-3}$	OXYGEN CONSUMPTION		
			Without azide	With azide	Resting muscle
			cmm./g./hr.	*cmm./g./hr.*	*cmm./g./hr.*
KCl	0.116M	2.3	60	19	
KCl	0.116M	2.1	74	30	27
KCl	0.116M	3.3	80	26	34
Acetyl choline	0.002M	1.9	78	34	28

oxygen consumption (stims. D and E, fig. 3). In most cases the contracture (see p. 198) renders it impossible to stimulate the muscles soon after the azide is added. Calculations of the "extra oxygen consumption" after azide showed no more than 5 per cent of that obtained after similar stimulation in the control period. Thus there was no extra oxygen consumed and no oxidative recovery after a tetanic stimulus sufficient to yield a marked effect in the control. Yet the call for extra oxygen must have been urgent.

Muscles which had failed to respond to stimulation could be restored to normal with regard to both irritability and extra O_2 uptake following stimulation by washing in Ringer's solution providing the exposure to azide was not too long. After a critical point in time of exposure, which is rather evanescent the muscle proceeds into rigor regardless of efforts to revive it.

5. *Caffeine.* Meyerhof (1921) and Hartree and Hill (1924) have hypothesized that caffeine in suitable concentrations releases continuously the

processes normally set off by electrical stimulation. In a recent study, Saslow (1937) has confirmed the large increases in oxygen consumption and lactic acid production in caffeine-treated muscles indicated by earlier work and finds his data consistent with the above hypothesis. In the work reported in this paper "stimulation" by caffeine presents one great advantage over the other forms used, viz., the "stimulation" is continuous and results in a continuously high rate of oxygen consumption which is practically constant with time. Also the new levels of respiration reached in the presence of inhibitor are constant.

To muscles respiring in the presence of 0.04 per cent caffeine in Ringer's

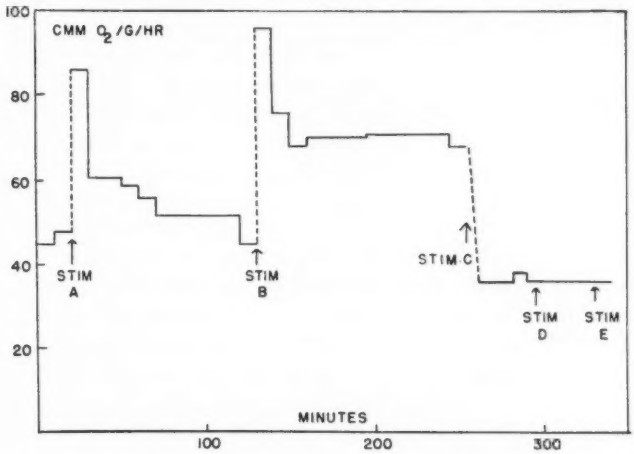

Fig. 3. Effect of azide on excess O_2 consumption after electrical stimulation (Thyratron stimulator). Stim. A = 15 sec. tetanus; stim. B = 30 sec. tetanus; stim. C = 30 sec. tetanus, tip azide ten seconds later; stim. D and E = 30 sec. tetanus, muscles responded, but no increase in O_2 consumption followed. Final concentration of azide 0.002M.

solution, azide solutions (made up in caffeine-Ringer) over a wide range of concentrations could be tipped. Within 10–20 minutes after tipping a new level of oxygen consumption was reached which remained constant with time for from one to four hours. This was true at all azide concentrations which exerted any effect at all. A typical experiment with caffeine is shown in figure 4. With such well-defined levels of respiration for computations a rather extensive series of experiments was possible and the results strengthen considerably the previously detailed considerations.

It soon became apparent that the maximum inhibition obtainable even at nearly M/10 azide was only about 75 per cent of the rate of oxygen

consumption in caffeine alone. This level in terms of cmm./g./hr. fell precisely within the range of the average resting rates (fig. 1). Between approximately 10^{-3} and 10^{-5} M the inhibition decreased progressively with decreasing concentration. Below 10^{-5} M the poison exerted practically no effect. Thus over a hundred-fold range of concentration (10^{-3} to 10^{-1}M) practically no further decrease in respiration took place, and, with regard to rates of respiration, the azide-insensitive fraction in caffeinized muscle falls precisely within the limits of the resting respiration.

The fact that a new constant level of O_2 consumption was reached for each concentration of azide indicated that perhaps a true equilibrium state might exist. If true, the data should fit a simple mass law formulation

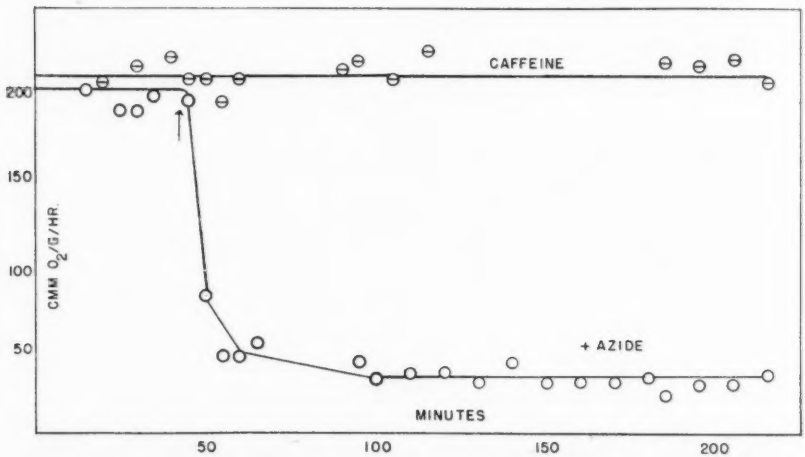

Fig. 4. Oxygen consumption in 0.04 per cent caffeine in Ringer's with and without azide. Open circles = experimental; barred circles = control. The azide (final concentration 0.0021 M) was tipped at the arrow.

when properly corrected for the azide-insensitive fraction. This was found to be actually the case and was chosen as the best quantitative method for presenting the results.

The simplest assumptions were made, viz. that the poison combines with some single enzyme which can be designated as E, and that the rate of respiration is proportional to the concentration of uncombined enzyme. Thus,

(1) $$[E] + C\,[Az] \rightleftharpoons [E\,Az_c]$$

(2) $$\frac{[E] + [Az]^c}{[E\,Az_c]} = k = \frac{n\,[Az]^c}{1 - n}$$

where $[E]$ = concn. enzyme, $[Az]$ = concn. azide, n = fraction of respiration not inhibited, $1-n$ = fraction inhibited and k = equilibrium constant. Then

$$\frac{1-n}{n} = k\,[Az]^C \tag{3}$$

and for convenience in plotting,

$$\log\ 1-n = C \log [Az] + \log k \tag{4}$$

If $\log 1-n/n$ be plotted against $\log [Az]$ a linear relation should result if the data fit this formulation, the slope of the line representing C, a measure of the number of molecules of poison combining with a single active enzyme group (i.e., the coefficient of $[Az]$ in equation 1, the exponent in equation 2). This type of formulation has been employed recently for the effects of cyanide (Fisher and Ohnell, 1938) and azide (Armstrong and Fisher, 1938) on embryo fish hearts.

In figure 5 the uncorrected data for caffeinized muscle are plotted in this manner. As expected from the fact that complete inhibition did not occur the line flattens off at about 10^{-3} M azide. The uncorrected data are thus not a proper test for the law, but are presented for comparison with the corrected. The slope of the diagonal line is 0.83, which is, however, without much significance since it probably represents a mixed metabolism. The same data corrected for the resting respiration on the assumption that this persists unchanged in the presence of caffeine are also plotted in figure 5. There seems little doubt that the best relation is a linear one and the line fitted has a slope equal to one. Thus it appears that between 10^{-5} and slightly above 10^{-3} molar concentrations sodium azide inhibits the increment in oxygen consumption caused by caffeine in a manner which superficially at least obeys the law of chemical equilibrium with one molecule of inhibitor inactivating one active enzyme group. In addition the data indicate that in all probability the resting metabolism persists unchanged in caffeinized muscle, and is a qualitatively distinct system.

Measurements of the anaerobic glycolysis showed that azide does not interfere with lactic acid production in caffeinized muscle. For example, two sets of muscles treated with 0.002 M azide in caffeine-Ringer's yielded average rates of CO_2 production of 508 and 537 cmm. CO_2/g./hr.; caffeinized muscles alone, 517; and resting muscle, 22. There seems little doubt but a similarly high rate of lactic acid production proceeds aerobically, since the pH fell rapidly after the first hour in azide solutions in O_2. Saslow (1937) has shown that caffeinized muscles which had been allowed to accumulate lactate in nitrogen reoxidized this lactate when placed in

oxygen. In my experiments the azide-treated caffeinized muscles accumulated lactate much more rapidly than did controls. It thus seems probable that the azide simply prevented oxidation of the excess lactate.

6. *Muscle mash.* Sodium azide quickly inhibits the high rate of oxygen consumption of muscle mash. For example, in a typical experiment the respiration of the control averaged 120 cmm. O_2/g./hr. for the first hour

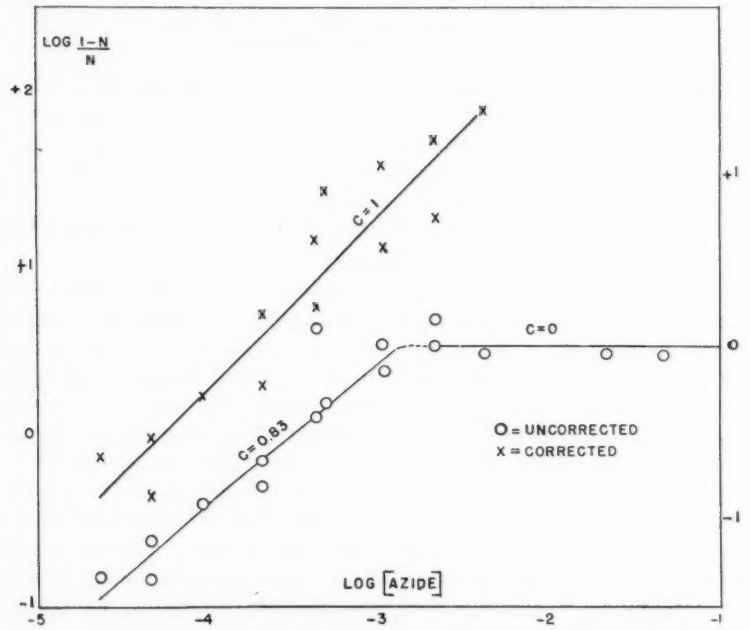

Fig. 5. Data for caffeinized muscle plotted according to equation 4 (see text). The lower line (circles) shows the relation between log $1-n/n$ and log [azide] when the data are plotted directly. The value of n is in each case the rate of respiration in azide; $1-n$ represents the difference between n and the rate in caffeine alone. The upper line (crosses) represents the relation when the raw data are corrected for the resting respiration by subtracting the average resting rate from both n and the measured respiration in caffeine alone. Thus total respiration = resting respiration + uninhibited caffeine fraction + inhibited caffeine fraction.

while in the presence of 0.002 M azide the rate of O_2 uptake averaged 45 cmm./g./hr. for the first hour after adding the poison. This level was reached within 20 minutes. In the second hour the control averaged 100 cmm./g./hr.; the azide-treated mash 34 cmm./g./hr. In the third hour the figures were 75 cmm./g./hr. for control and 22 cmm./g./hr. for azide. Although the levels established were not as clear-cut as those

characteristic of intact muscle, the fact that the increased O_2 consumption of a mash is probably due to lactate formation (Meyerhof, 1920) and the finding here that this increase is largely inhibited by azide suggest again that this inhibitor prevents oxidation of lactate as it is formed.

7. *On the mechanism of action of azide.* Keilin (1936) has shown beyond a reasonable doubt that sodium azide inhibits the action of the "indophenol oxidase." The most likely action in muscle would then be on the War-

TABLE 3

Typical experiment showing effect of NaN_3 on the oxidation of dimethyl-paraphenylenediamine (PPD)*

RESPIROMETER	OXYGEN CONSUMPTION			
	A	B	C	D
	cmm./g./hr.	*cmm./g./hr.*	*cmm./g./hr.*	*cmm./g./hr.*
Control period	38	44	31	35
	Tip PPD-azide	Tip PPD-azide	Tip PPD	Tip PPD
First hour	50	52	155	143
Second hour	36	48	89	96

* Concentration 8.6×10^{-3} M.

TABLE 4

Summary of experiments with para-phenylenediamine (PPD) and with methylene blue (MB)

SUBSTANCE ADDED	CONCENTRATION M	CONCENTRATION AZIDE M × 10^{-3}	OXYGEN CONSUMPTION		
			Without azide	With azide	Resting
			cmm./g./hr.	*cmm./g./hr.*	*cmm./g./hr.*
PPD	1.0×10^{-3}	2.4	106	44	25
PPD	3.7	2.1	109	63	35
PPD	2.2	2.2	100	43	29
PPD	4.3*	8.6	149	51	33
MB	4.1×10^{-5}	2.0	92	87	50
MB	4.2	2.1	87	77	32

* Dimethyl-p-phenylenediamine.

burg-Keilin oxygen transfer system. One method of approach to this problem is to determine the influence of the inhibitor on the oxidation of p-phenylenediamine. Recent work of Stotz, Sidwell, and Hogness (1938) indicates that this compound is oxidized by both the cytochrome C-cytochrome oxidase complex and autoxidizable cytochrome B. Only the former catalysis is inhibited by cyanide. Tables 3 and 4 show that azide at a concentration sufficient to eliminate almost completely any extra O_2 uptake due to contraction by electrical or chemical stimulation

causes a large but not complete inhibition of the oxidation of PPD. The results are qualitatively, at least, those which would be expected if the azide were acting by inhibition of the cytochrome C-cytochrome oxidase complex, with a residue of oxidation attributable to cytochrome B.

Another method of attack is to "short-circuit" the Warburg-Keilin oxygen transport system by the addition of autoxidizable dyes such as methylene blue or pyocyanin (Barron and Hastings, 1933). As shown in table 4, the large increase in the respiration of muscle due to the presence of methylene blue is not inhibited appreciably by 0.002 M azide. The data given are for the first hour. In the second and third hours the rate in azide plus methylene blue fell with time while that with the dye alone

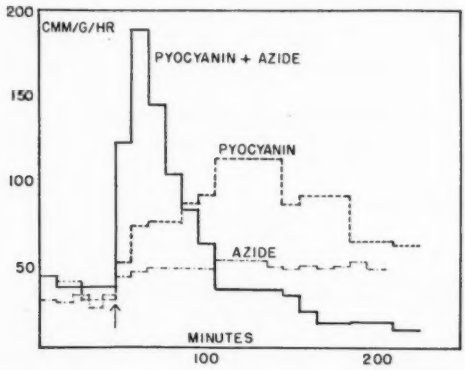

Fig. 6. Oxygen consumption in pyocyanin, pyocyanin plus azide, and azide alone. At arrow pyocyanin alone (final concentration 0.005 per cent) was tipped onto one set of muscles (broken line) while pyocyanin plus azide (final concentration of azide 0.002M) was tipped onto the other set of muscles (solid line). The data for azide alone are from another experiment, but are representative.

remained constant. With pyocyanin the initial effect of azide was a large increase in the rate of respiration followed by inhibition, both accompanied by contracture. This is illustrated in figure 6. While this latter case requires further elucidation,[2] it is clear that initially the O_2 consumption due to either carrier is not inhibited by azide in the manner characteristic of the increased metabolism associated with stimulation of the contractile mechanism.

[2] The initial increase which appeared after tipping pyocyanin plus azide (fig. 6) was apparent also, but to a lesser degree, with methylene blue. In both cases it may be due to the fact that a carrier was present to enable connection between the oxidizable substrates produced by the contracture in azide and molecular oxygen. The later inhibitions may be attributed to secondary effects whose nature is now under observation.

The results obtained by either mode of attack are consistent with the idea that this poison prevents the extra oxygen consumption associated with activity by inhibiting the Warburg-Keilin oxygen transport system. Since azide also inhibits catalase (Keilin and Hartree, 1936) it is possible that its action should be attributed to this property. However a direct rôle of catalase in cellular respiration has not been demonstrated and the inhibitions occur here within a very short time. This alternative is considered much less likely.

8. *On the action of cyanide.*[3] If the action of azide is on the cytochrome-cytochrome-oxidase system then the resting respiration must not traverse this system. Yet, as indicated earlier, the resting oxygen consumption is inhibited by cyanide. Experiments were arranged to study the effect of cyanide on resting and caffeinized muscle. It was apparent that the respiration in either case could be completely inhibited by cyanide, but a larger fraction was inhibited at moderate cyanide concentrations in the caffeinized muscle. The levels reached at any given cyanide concentration were regular enough so that the same mass law formulation was applied as in the case of azide. The values for C obtained by plotting $\log 1-n/n$ against $\log [CN]$ are different for resting and caffeinized muscle, the caffeinized muscle having the higher value (fig. 7). If the data for caffeinized muscle are "corrected" the difference is more evident. For the "corrected" curve the values of m are the measured rates for caffeinized muscle in cyanide minus the rate of O_2 uptake in resting muscle at the same concentration of cyanide, while $1-n$ represents the difference between this figure and the increment in respiration caused by caffeine. C for resting muscle is 0.84 while that for caffeinized muscle becomes 1.2.[4] These data are interpreted as indicating that while both resting and caffeinized oxygen uptakes are inhibitable by cyanide the kinetics are sufficiently different to indicate that cyanide is inhibiting two qualitatively different fractions.

Discussion. An alternative explanation to a qualitative fractionation of the respiration might be one based on "unsaturation" of the enzyme surfaces. This view, first enunciated by Warburg (1927), states that at less than the maximum respiratory rate the oxidation enzymes may be "unsaturated" with substrate and consequently considerable portions of the enzyme may combine with poison without noticeable effect on the rate

[3] KCN-KOH mixtures were used to absorb the CO_2. Thanks are due to Miss Helen Abramowitz for assistance with some of these experiments.

[4] The theoretical meaning of the values 0.84 and 1.2 is not clear since the simple theory calls for whole numbers. However, fractional orders of reaction are not unknown in chemical kinetics. Values between one and two for the action of azide and cyanide on embryo hearts have been reported by Armstrong and Fisher (1938). Further study of this question is contemplated.

of respiration. While the original formulation of Warburg is probably incorrect (Fisher and Cameron, 1938) and there is no direct proof of the theory, there is a large body of literature on cellular respiration which can be interpreted qualitatively in this manner (Runnström, 1928; Friedheim, 1931; Cook, Haldane and Mapson, 1931; Orström, 1935; Stier and Stannard, 1936; Commoner, 1938; and others). However this explanation cannot apply to the lack of inhibition of the resting O_2 uptake by azide. First, the plateau reached in the relation of concentration to effect is too prolonged (100-fold range, fig. 1). Secondly, as shown in figure 2, lactic acid production is stimulated by the contracture and continues unhampered even in the presence of sufficient azide to prevent any increment in oxygen

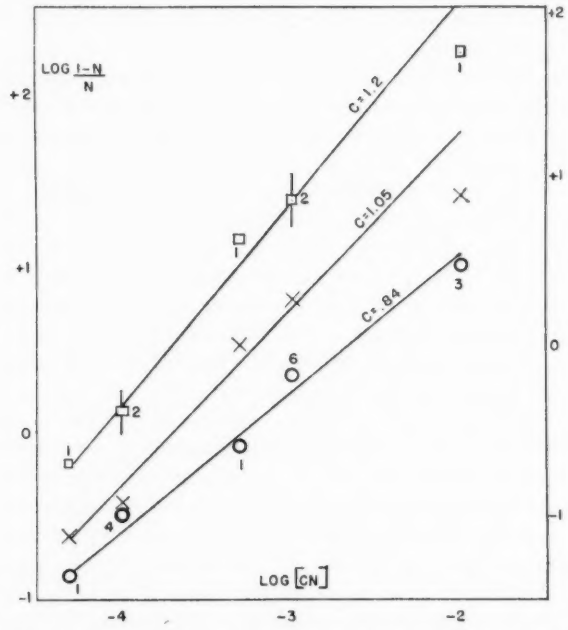

Fig. 7. The effect of cyanide on resting and caffeinized muscle plotted in the same manner as in figure 5 for azide. Open circles = resting muscle; crosses = caffeinized muscle, raw data; squares = caffeinized muscle corrected for the resting respiration as explained in the text. The points at 10^{-2} M for caffeinized muscle were given less weight in fitting the line since the inhibition is nearly complete and the rate of respiration almost within the experimental error of the method. The numbers affixed to the points represent the number of experiments contributing to each point. The height of the bars on the upper curve indicates the spread of points in the experiments with caffeinized muscle. For resting muscle the range was of the same order as shown in figure 1.

consumption on stimulation (fig. 3). There is ample substrate present to support much higher rates of oxidation than those characteristic of resting muscle. Hence "unsaturation" of a single enzyme system cannot account for the difference between resting and active muscle in azide. Similarly, lactic acid production is unhampered by cyanide (Meyerhof, 1923) and was proceeding even in the resting muscle after cyanide treatment. Thus neither the azide nor cyanide observations can be explained on the basis of "unsaturation."

Ross[8] has found recently (1938) that he could fit the observed inhibitory effects of cyanide on the oxygen consumption of wheat seeds to equations in which one molecule of cyanide combined with three of respiratory enzyme. Regardless of whether or not his data actually fit the equations, the theory indicated that in this case it was not necessary to postulate a cyanide-stable fraction of the respiration to explain lack of complete inhibition by cyanide. However the analysis presented in figure 5 shows that in the present experiments with azide a single system could not be easily postulated. The relation between effect and concentration of poison is quite regular with an apparent ratio of one azide molecule to one enzyme molecule (or group) up to complete inhibition of that fraction. From that concentration on there is no effect at all (excluding secondary effects). Unless it be assumed that application of the mass law concept is entirely erroneous it is difficult to explain this discontinuity on the basis of a single system.

On the basis of the present experiments it appears that frog muscle does not make use of the cytochrome system until stimulated. In many respects this parallels observations on the activation of sea-urchin eggs (Korr, 1937), in which cytochrome is "thrown into circulation." In muscle, on the classical viewpoint, the primary change would probably not be in the oxygen transferring mechanism, but instead the cytochrome system may simply lack substrate until such is produced by the glycogenolytic system. If oxidation is direct (Sacks, 1938) then a primary change may include this one. Data taken under less specialized conditions are necessary to shed light on this point. However it is clear that if the resting respiration is a qualitatively separable system it is correct to assume too, that only the "extra" oxygen consumption is involved in recovery from stimulation (Burk, 1937). Other applications of such a fractionation to general conceptions of muscular activity are obvious.

SUMMARY

1. Sodium azide has no immediate inhibitory effect on the oxygen consumption of resting frog muscle over a ten-thousand fold range of concentration. No marked change in effect occurs on lowering the pH, except a slightly more rapid decline in respiratory rate after the first hour.

2. The increment in O_2 uptake caused by electrical stimulation, KCl or acetylcholine contracture, or treatment with sub-contracture doses of caffeine is completely eliminated by 0.002 to 0.005 M azide. The inhibition takes place within a few minutes.

3. The effects on the O_2 consumption in caffeine indicate that the resting respiration continues unhampered in the presence of the much higher rates associated with caffeine treatment. The azide-sensitive fraction behaves as if it represented a true chemical equilibrium between enzyme and inhibitor with one molecule of inhibitor combining with one active enzyme group.

4. The oxidation of p-phenylene-diamine is almost but not completely inhibited, while the increased O_2 consumption due to the addition of methylene blue or pyocyanin is not prevented until secondary effects supervene. This indicates that the azide is probably inactivating the Warburg-Keilin oxygen transport system.

5. Azide has no inhibitory effect on glycolysis.

6. In the resting muscle a small contracture attends the action of azide.

7. The effects of cyanide on resting and caffeinized muscle indicate that it may be affecting two different enzyme systems.

8. It is proposed that the respiration of frog muscle can be separated into two distinct fractions—one which functions only in activity and the other characteristic of resting muscle. Both fractions are inhibited by cyanide but with different kinetics, while only the former is sensitive to azide. In addition, it appears that the Warburg-Keilin (cytochrome-cytochrome oxidase) system functions only in activity of the muscle.

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